University of Dundee

MASTER OF SCIENCE

Regulation of NEDD8/Rub1 and its substrates

Curran, Siobhan

Award date:
2013

MASTER OF SCIENCE

Regulation of NEDD8/Rub1 and its substrates

Siobhan Curran

2013

University of Dundee

Regulation of NEDD8/Rub1 and its substrates

By: Siobhan Curran

Supervisor: Thimo Kurz

A thesis submitted for the degree
Master of Science,
College of Life Sciences,
University of Dundee, February 2012

Table of Contents

List of Figures

List of Tables

Acknowledgements

I would like to thank my family and friends. I would also like to thank my supervisor Thimo Kurz and members of the Kurz lab for assistance: Aleksandra Zemla and Sylwia Kedziora for assistance with yeast techniques, Yann Thomas for assistance with tissue culture and FACS analysis, and Roland Hjerpe for assistance with tissue culture and the NEDD8 affinity matrix.I would also like to thank the people who have provided support and assistance including Angela Nicoll, Letitia Gibson, Kirsten McLeod, Hannah Kendall,and Rachel Naismith. I would also like to thank members of the Protein Production and Assay Development Team, Axel Knebel, Richard Ewan, Daniel Fountaine, Clare Johnson, and Anees Ahmed Basha. The DNA cloning service was also immensely helpful: Nicola Wood, Melanie Whitman, Tom Macartney, and Zoe Gage. I would also like to thank the Oligonucleotide Synthesis Group and the DNA sequencing group. I also want to thank all of the people who helped me with mass spectrometry including the members of Patrick Pedrioli's Laboratory: Patrick Pedrioli, Kshitiz Tyagi, and Van Kelly, and the members of the Proteomics and Protein Chemistry Service including Matthias Trost, David Campbell, Stella Ritorto, Brian Dill, Bob Gourlay, and Joby Varghese. I would also like to thank Hilary McLauchlan and James Hastie of the Division of Signal Transduction Therapy for antibody production and Natalia Shapiro for MLN4924 synthesis.I would like to thank Esther Sammler, Susanne Bandau, Brian Dill, Francisco Inesta-Vaquera, Vanessa Houde, and Ana Belen Perez-Oliva for mouse tissues.Lastly, I would also like to thank the staff that took care of our glassware, rubbish, and cleaning.

Declarations

I hereby declare that this dissertation is the product of my own investigation and this thesis is of my own composition. Where contributions were made in whole or in part by others I have indicated in the text by reference to the researchers or publications. This dissertation has not been presented in whole or in part for a higher degree.

Siobhan Curran

I confirm that I have read and approved this work and that the above is true to the best of my knowledge.

Thimo Kurz

Abbreviations

Δ	Deletion
ΔGG	Deletion of the diglycine motif (nonconjugateable)
°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
3' UTR	3' untranslated region
5' UTR	5' untranslated region
Å	Angstrom
A_{260}/A_{280}	Absorbance at 260 nm/ Absorbance at 280 nm
ABC	Ammonium bicarbonate
ACN	Acetonitrile
AD	Alzheimer's Disease
AICD	APP intracellular domain
AMP	Adenosine monophosphate
amp	Ampicillin (we used carbenicillin from Formedium)
APP	Amyloid precursor protein
APPBP1	APP binding protein 1
APS	Ammonnium persulfate
Atg12	Autophagy-12
Atg8	Autophagy-8
ATP	Adenosine triphosphate

BCA	Bicinchoninic acid
BCA3	Breast cancer associated gene 3
Bis-tris	2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol
BLAST	Basic local alignment tool
bp	Base pair
BRE1	Brefeldin A sensitivity 1
BSA	Bovine serum albumin
C	Celsius
C18	Carbon 18
CAND1	Cullin associated NEDD8-disocciated 1
Cdc53	Cell division cycle protein 3
cDNA	Complementary DNA
CO_2	Carbon dioxide
COP	Coatomer
COP9	Constitutive photomorphogenesis protein 9
CPS	Counts per second
CRL	Cullin RING ligase
Crt10	Constitutive RNR Transcription regulator 10
CSN5	COP9 signalosome complex subunit 5
C-terminal	Carboxy terminal
C-terminus	Carboxy terminus
CUL	Cullin
Dcn	Defective in cullin neddylation

DH5α	*E. coli* strain
diglycine motif	C-terminal Glycine 75/76 of ubiquitin or NEDD8
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered sodium
DSS	Disuccinimidyl suberate
DTT	dithiothreitol
DUB	Deubiquitylating enzyme
E. coli	*Escherichia coli*
E1	E1 activating enzyme
E2	E2 conjugating enzyme
E2-25K	Ube2k
E3	E3 ligase
ECL	Enhanced chemiluminescent reagent
EDTA	ethylenediamine tetracetic acid
EGFR	Epidermal growth factor receptor
Epitomics anti-NEDD8	Rabbit monoclonal anti-NEDD8 antibody from Epitomics
ERAD	Endplasmic reticulum associated degradation
FACS	Fluorescence activated cell sorting

FAT10	Human leukocyte antigen-F associated transcript 10
FBS	Fetal bovine serum
g	gram
G1	Gap1 phase
G2/M	Gap2 phase/mitosis phase
G75/76	Glycine 75 and 76 (diglycine motif) of NEDD8 and ubiquitin
G75/76A	Diglycine motif mutated to dialanine
G75/76V	Diglycine motif mutated to divaline
G76	Glycine 76 of ubiquitin or NEDD8 (preferentially used for conjugation)
GAL promoter	Galactose inducible promoter
GFP	Green fluorescent protein
GO	Gene ontology
GST	Glutathione S-transferase
h	hour
HA	Hemagglutinin tag
HCl	Hydrogen chloride
HCT-116	Human colon carcinoma cell line 116
HECT	Homologous to the E6-AP carboxyl terminus
HEK293	Human Embryonic Kidney 293 cells
HeLa	Human cervical cancer cell line
His3	Histidine 3
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-

	Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
Hif	Hypoxi- inducible factor
HNRNPK	Heterogenous ribonucleoprotein K
HRP	horseradish peroxidase
IAA	Iodoacetamide
IgG	Immunoglobulin G
IP	Immunoprecipitate
Isg15	Interferon-stimulated gene-15
JAMM	Jab1/MPN/Mov34 metalloenzyme domain
k	Kilo
KAN	Kanamycin cassette
kb	Kilobase pair
kD	Kilodalton
KO	knock out
L	Litre
Laemmli	3 x reducing sample buffer
Lag2	Longevity assurance gene 2
LB	Lysogeny broth
LC-MS-MS	Liquid chromatography tandem mass spectrometry
Leu2	Leucine biosynthesis 2
LiAc	Lithium acetate
m	Milli
M	Molar

MDM2	Murine double minute 2
MES	2-(N-morpholino)ethanesulfonic acid
mg	Milligram
MG132	*N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal
Mil10	Rabbit monoclonal anti-NEDD8 from Millenium Pharmaceuticals
MilliQ	MilliQ ultra pure water
min	Minute
MJD	Machado-Josephin domain
mL	Millilitre
MLN4924	[(1S,2S,4R)-4-[4-[[(1S)-2,3-dihydro-1H-inden-1-yl]amino]-7H-pyrrolo[2,3-d]pyrimidin-7-yl]-2-hydroxycyclopentyl]methyl ester
mM	Milli molar
MMS1	Methyl MethaneSulfonate sensitivity 1
MOPS	3-(N-morpholino)propanesulfonic acid
MRC	Medical Research Council
mRNA	Messenger RNA
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
MW	Molecular weight
NAM	NEDD8 affinity matrix
N8	NEDD8
NAE	NEDD8 activating enzyme
NEDD8	Neurologically expressed developmentally downregulated 8

NEDP1	NEDD8 specific protease 1
NEDP1 C163A	Catalytically dead NEDP1
N-end rule	Proteins bearing destabilizing N-terminal residues are degraded quickly
ng	Nanogram
NHS	N-hydroxysuccinimide
nM	Nanomolar
nm	nanometer
NMR	Nuclear magnetic resonance
NONO	Non-POU domain-containing octamer-binding protein
NP-40	Nonidet p40
N-terminal	Amino terminal
N-terminus	Amino terminus
NTKL	N-terminal kinase like protein
NUB1	NEDD8 ultimate buster 1
OD	Optical density
OD_{600}	Optical density at 600 nm
OPT	1,10-Phenanthroline
OTU	ovarian tumour domain
p53	Tumour protein 53
p63	Tumour protein 63
p73	Tumour protein 73

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdr5	Pleiotropic Drug Resistance 5
PEG	Polyethylene glycol
Pep4	CarboxyPEPtidase Y-deficient
pH	Potential for hydrogen
PPAD	Protein production and assay development group
PTM	Posttranslational modification
pVHL	Von Hippel-Lindau protein
Rabbit anti-NEDD8	Polyclonal rabbit anti-NEDD8 antibody
Rbx	RING-box protein
Rfu1	Regulator of Free Ubiquitin chains 1
RING	Really interesting new gene
RNA	Ribonucleic acid
RNAi	RNA interference
Rri1	Regulator of Rub1 specific isopeptidase
RT	Room Temperature
Rtt101	Regulator of Ty1 Transposition 101
Rub1	Related to ubiquitin 1
Rub1 pr.	5' UTR
S. cerevisiae	*Saccharomyces cerevisiae*
Saccharomyces	*Saccharomyces cerevisiae*

San1	Sir Antagonist 1
SCF	Skp1, cullin, F-Box complex
SCILLS	Scottish Institute for Cell Signalling
SD	Synthetic dextrose media
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide electrophoresis
SFPQ	Splicing factor, proline- and glutamine-rich
SGD	*Saccharomyces* genome database
Sheep anti-NEDD8	Polyclonal sheep anti-NEDD8 antibody
Sic1	Substrate/Subunit Inhibitor of Cyclin-dependent protein kinase
SILAC	Stable isotope labelling of amino acids in cell culture
siRNA	Small interfering RNA
Skp1	Suppressor of Kinetochore Protein mutant 1
SMC	Structural maintenance of chromosomes
SpeedVac	Centrifugal evaporator
Ssa	Stress seventy subfamily A
SUMO	Small ubiquitin-like modifier
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TCA	Trichloro acetic acid (used to precipitate proteins)
TDRD7	Tudor domain containing protein 7
TE	Tris EDTA

TEABC	Triethyl ammonium bicarbonate
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TGS	Tris glycine SDS buffer
TPP	Trans Proteomic Pipeline
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Trp1	TRyPtophan requiring 1
TS	Temperature sensitive
TUBES	Tandem-repeated ubiquitin-binding entities
U2OS	Human osteosarcoma cell line
UAE	ubiquitin activating enzyme
Ub	Ubiquitin
UBA1	Ubiquitin-like modifier-activating enzyme 1
UBA1Y	Ubiquitin-like modifier-activating enzyme 1Y (testes specific isoform)
Uba3	Ubiquitin-like protein-activating enzyme 3
Uba52	Ubiquitin ribosomal fusion
UBA6	Ubiquitin-like modifier-activating enzyme 6
Uba80	Ubiquitin ribosomal fusion
Ubb	Polyubiquitin B
UBB+1	Mutant misread ubiquitin with 19 residue C-terminal extension
Ubc	Ubiquitin-conjugating enzyme
Ubc4/5	Ubc4 and Ubc5

Ubc4/5/6/7	Ubc4, Ubc5, Ubc6, and Ubc7
Ubc6/7	Ubc6 and Ubc7
Ube	Polyubiquitin C
UBE1	Ubiquitin-like modifier-activating enzyme E1
UBE2K	Ubiquitin-conjugating enzyme E2 K
Ubi1	Ubiquitin ribosomal fusion (*S. cerevisiae)*
Ubi2	Ubiquitin ribosomal fusion (*S. cerevisiae)*
Ubi3	Ubiquitin ribosomal fusion (*S. cerevisiae)*
Ubi4	Ubiquitin 5 tandem repeats (*S. cerevisiae)*
UBL	Ubiquitin like protein
UBP	Ubiquitin specific protease domain
Ubr1	Ubiquitin protein ligase E3 component n-Recognin 1
UCH	Ubiquitin carboxy-terminal hydrolase domain
UCHL1	Ubiquitin carboxy-terminal hydrolase isozyme L1
UCHL3	Ubiquitin carboxy-terminal hydrolase isozyme L3
Ula1	Ubiquitin-like activating-activating enzyme
ULP	Ubiquitin like protease domain
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
URA	Uracil
Urm1	Ubiquitin related modifier-1
USP	Ubiquitin specific protease domain
USP21	Ubiquitin specific protease domain protein 21

V	Volts
v/v	Volume/volume
w/v	Weight/volume
WB	Western Blot
WT	Wild type
X g	Times gravity
XIAP	X-linked inhibitor of apoptosis
yeast	*Saccharomyces cerevisiae*
YPAD	Yeast extract-peptone-dextrose-plus Adenine
Yuh1	Yeast ubiquitin hydrolase

Amino Acid Code

Amino acid	Three letter code	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Cullin molecular weights by species

Cullin	Human (kD)	Mouse (kD)	Yeast (kD)
cullin 1 (Cdc53)	89.7	89.7	93.94
cullin 2	87	86.9	
cullin 3 (Cul3)	88.9	88.9	86.11
cullin 4a (Rtt101)	87.7	87.8	99.33
cullin 4b (Rtt101)	104	110.7	99.33
cullin 5	100	100	
cullin 7	191.2	192.3	
cullin 9	281.2	209.2	

Yeast cullins are named parenthetically alongside their homologs. Cullin 9 is also commonly known as PARC.

Summary

NEDD8 is a small ubiquitin like modifier (UBL) essential for life in almost all known eukaryotes. The primary objective of this work is to determine NEDD8 specific regulatory mechanisms. Like ubiquitin, NEDD8 is first matured and then conjugated via a tripartite conjugation cascade (E1, E2, E3). Although Arg72 in ubiquitin prevents misactivation of ubiquitin by the NEDD8 E1, the ubiquitin E1 is not as selective(Souphron et al., 2008). Under conditions of stress or NEDD8 overexpression, the ubiquitin E1 can be charged with NEDD8(Hjerpe et al.; Hjerpe et al., 2012b; Leidecker et al., 2012). Most ubiquitin E2s can accept the aberrantly activated NEDD8which then ultimately becomes conjugated to substrates that are normally ubiquitylated(Hjerpe et al., 2012a). Consequently, maintaining the ratio of NEDD8 to ubiquitin is important for maintaining the integrity of the ubiquitin pathway.

The mechanism by which the ubiquitin to NEDD8 ratio is regulated remains unclear. In order to insulate the ubiquitylation pathway from NEDD8, we postulated that the ratio of free NEDD8 to free ubiquitin would be tightly controlled. The rapid turnover reported to exist for NEDD8(Hipp et al., 2004) could be one means of regulating that ratio. However, we have discovered that in yeast that tagging the NEDD8 homolog, related to ubiquitin 1 (Rub1) results in abnormally fast degradation through the unfolded protein response (UPR). Endogenous Rub1 is actually quite stable. The pathway by which endogenous NEDD8 is degraded remains unknown.

Another way of distinguishing the two pathways and uncovering NEDD8specific regulation is to identify genuine substrates of the neddylation pathway. We were unable to uncover novel substrates in yeast. We used several methods to enrich and identify NEDD8 substrates in mammalian cells including cultured human cancer cells and wild type mouse tissue.

Mouse testeshave an increased neddylation profile by Western Blot (WB). We therefore attempted to identify NEDD8 substrates in mouse testes. We examined both the NEDD8 associated proteome and NEDD8 conjugates by using native and denaturing immunoprecipitation experiments paired with mass spectrometric (MS) analysis. First, we are confident in these experiments because we identified NEDD8 itself. We also observed the cullin family of proteins which have been well characterized as the main substrates of neddylation. One surprising find in the NEDD8 associated proteome was cullin associated, NEDD8 dissociated 1 (CAND1) which binds to the cullins and precludes NEDD8 conjugation to the cullins. We show that CAND1is unneddylated itself but comes down in association with neddylated proteins. Interestingly, we also found the ubiquitin E1associated with NEDD8, indicating that there may be endogenous charging of the ubiquitin E1with NEDD8 at low levels.The only directNEDD8 conjugates well established in this work were the cullins. Further work is needed to completely rule out the existence of non-cullin substrates of the neddylation machinery.

Chapter 1: Introduction

It is through the presence, absence, structural change, and modification of proteins that life is capable of functioning. Posttranslational modifications (PTMs) such as neddylation, ubiquitylation, and phosphorylation allow for dynamic regulation of protein functions such as translating genetic code, coordinating responses to changes in the environment, recognizing and propagating signals from outside the cell and serving in cellular transportation networks. In order to control these molecular switches, PTMs are themselves regulated. For example, NEDD8, the small protein responsible for neddylation is synthesized in an inactive form and requires the activity of several enzymes before it can modify its eventual substrate.

1.1 Protein turnover

There is a constant flux betweenthe synthesis of new proteins and the degradation of old proteins. This balance is carefully and specifically regulated to prevent conflicting signals from coexisting and to keep nonfunctional proteins from damaging overall cellular health. The importance of this system is highlighted by the fact that the accumulation of proteins that would normally be degraded is a hallmark of many neurodegenerative diseases such as Alzheimer's Disease (AD)(Upadhya and Hegde, 2007) and Parkinson's Disease(Lim and Tan, 2007), and neurological insults such as ischemic strokes(Lim and Tan, 2007). While it can be difficult to determine whether such accumulation is the causative agent or a consequence of these diseases, it is known thatthe aggregation of these proteins can further exacerbate the inhibition of the degradation machinery(Howlett, 2003). The degradation of key cell cycle and cell death regulators also plays an important role in cell survival, propagation, and growth, so

misregulation of degradative machinery is associated with many types of cancers(Edelmann et al., 2011). For this reason, the ubiquitin system is a growing target of chemotherapeutic research and the study of degradation is a burgeoning field for those interested in a range of health issues(Chauhan et al., 2005).

1.1.1 Lysosomal degradation

There aretwo main protein degradation pathways. First, lysosomal degradation is mediated by highly conserved acidic membrane bound organelles packed with proteases, lipases, and nucleases known as the lysosome (or vacuole in plants and yeast). Lysosomes are capable of processing complex mixtures taken in by the cell from its surroundings, whole organelles from within its boundaries, and even some specific internal proteins. Lysosomes are large enough to accomplish the degradation of old or damaged organelles and nonspecific enough to perform proteolysis on a diverse array of substrates.The acidic (pH ~5) membrane bound lysosome/vacuole protects the rest of the cell from the harsh environment necessary for both the activation of the specific lysosomal proteases (which function best in acidic environments) while simultaneously disrupting the structures and inactivating the proteins destined for degradation (Ciechanover, 2005).

1.1.2 Ubiquitin Proteasome System (UPS)

1.1.2.1 UPS: overview

The second important pathway in protein turnover, the ubiquitin proteasome system (UPS),is much more specific. Proteins to be degraded via this route are tagged with ubiquitin,an 8.5 kD protein called first discovered in 1975. Ubiquitylation (alternatively

called ubiquitination/ubiquitinylation) of substrate proteins can result in a variety of outcomes including endocytosis, DNA repair, activation of protein kinases, nuclear export, and substrate degradation. The importance of ubiquitin regulated degradation was acknowledged when Aaron Ciechanover, Avram Hershko, and Irwin Rose were awarded the Nobel Prize in Chemistry in 2004 for their work with ubiquitin-mediated proteolysis (The Nobel Prize).

While ubiquitylation may in a few instances result in degradation via the lysosomal pathway (Hicke, 1997), it is far more typical for ubiquitin modification to signal for substrate degradation via the ubiquitin proteasome system (UPS). The UPS is essential for viability and,when misregulated,is associated with many diseases. Typically, ubiquitin acts as a posttranslational modifier targeting substrates for degradation in a multisubunit cellular recycling plant known as the proteasome. The proteasome,through a series of 2 chymotrypsin-like, 2 trypsin-like, and 2 caspase-like proteolytic active sites, cleaves the proteins into 7-9-amino-acid long peptides.(Chauhan et al., 2005)

1.1.2.2 UPS: Importance of the system

Despite the inability of the proteasome to degrade whole organelles, and therequirement for ATP dependent ubiquitylation of individual substrate proteins, proteasomes are responsible for alarge amount of protein turnover. In fact, approximately 30% of newly synthesized cellular proteins are degraded by the proteasome after failing to pass through quality control checkpoints. In addition to these short-lived proteins, the UPS also processes the turnover of many other types of proteins such as cell cycle regulators,

pro-apoptotic proteins, and damaged proteins. In order to address the large volume of proteins that are regulated by this system, there are approximately 30,000 proteasomes in a single human cell. Furthermore the importance of this pathway is highlighted by its misregulation in many diseases and targeting by viruses (such as human immunodeficiency virus), and is essential for life because of its central role in the UPS. (Chauhan et al., 2005)

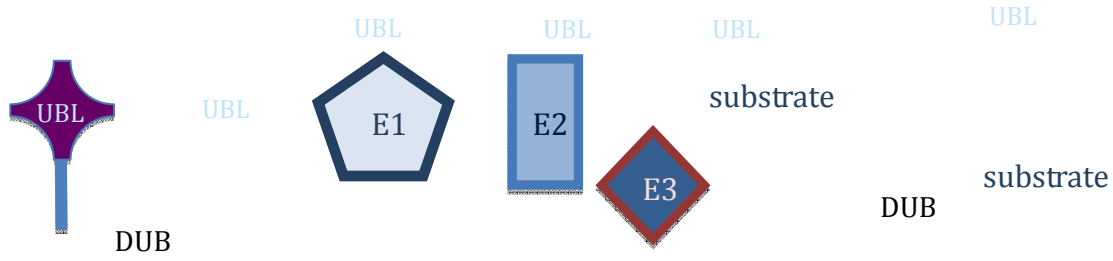


Figure 1.1. UBL conjugation cascade. UBLs are synthesized as inactive precursors that are matured by C-terminal cleavage revealing the diglycine motif(denoted by the green rectangle) which is then used in the conjugation cascade. The UBL is activated in an energy dependent step (denoted by the red arrow) and becomes covalently conjugated to the E1 activating enzyme. The UBL is then transthioesterified to the active site cysteine of the E2 conjugating enzyme. Then through the action of an E3 ligase the UBL becomes covalently conjugated to a substrate protein. Deconjugation is accomplished through the action of a deubiquitylating enzyme.

1.1.2.3 UPS: Ubiquitin

In order to posttranslationally modify substrates, ubiquitin followsa highly controlled mechanism (Figure 1.1). In mammals ubiquitin is encoded by four genestwo of which, *uba53*, and *uba80*, are ubiquitin ribosome subunit fusions and the remaining two, *ubb* and *ubc* are head to tail tandem ubiquitin pentamers whose expression is induced by cellular stress. Similarly in yeast, ubiquitin is synthesized fused to a ribosome subunit (Ubi1, Ubi2, Ubi3) or in cases of stress as a pentamer (Ubi4). Ubiquitin monomers are released from these fusion proteins to reveal a diglycine motif at the C-terminus.The

terminal glycine is utilized in conjugation to enzymes of the ubiquitylation pathwayand also eventually to substrate proteins. (Shabek and Ciechanover, 2010)

1.1.2.4 Ubiquitin conjugation cascade

Once monoubiquitin is released, it can proceed througha tripartite conjugation cascade (Figure 1.1). First,ubiquitin is activated by the ubiquitin E1 also known as the ubiquitin activating enzyme (UAE). In humans there are two ubiquitin E1 activating enzymes (UBA6and UBE1), in mice there is an additional testes specific isoform (UBA1Y). In yeast there is only one UAE (Uba1). The UAE functions by forming a high-energy ubiquitin-adenylate intermediate with the digylcine motif of ubiquitin in an ATP dependent step. AMP is released as a thiol-ester bond is formed between this charged ubiquitin and the UAE active site cysteine. A second ubiquitin is recruited to form a ubiquitin-adenylate intermediate such that there are two ubiquitin molecules simultaneously bound to the E1.

The E1thiol-ester linked ubiquitin is then transferred from the active site cysteine of the UAE to the active site cysteine of an E2 conjugating enzyme(E2) where it forms a thiol-ester bond. There are approximately 60 human E2 enzymes and 13 in yeast. A few E2 enzymes do not conjugate ubiquitin but rather ubiquitin like proteins (UBLs) such as SUMO or NEDD8.

The third enzyme in the conjugation cascade, the E3 ligase (E3), is responsible for conjugation of ubiquitin to substrate proteins. Some E3 enzymes, such asthose that function using a Homologous to the E6-AP Carboxyl Terminus (HECT) domain, directly bind ubiquitin and then transfer ubiquitin to substrates. Other E3s, such

ascontaining Really Interesting New Gene (RING) domains,never directly accept ubiquitin but rather provide structuralscaffolding to facilitate the transfer of ubiquitin from a ubiquitin charged E2 to the substrate protein. There are over 600 human RING family E3 enzymes. There are other much smaller E3 families including U-box domain containing proteins as well as the RING-between-RING family. By sheer force of numbers, E3s surpasses the number of kinasesin the human genome indicating the relative significance of this pathway. (Deshaies and Joazeiro, 2009)

1.1.2.5 Ubiquitin system specificity

There is a common misconception that substrate specificity for the ubiquitin system radiates out from the E1, with the E2 slightly narrowing the field of eventual substrates and the E3 providing ultimate specificity. The passage of ubiquitin to an E2 narrows the field of proteins that can be modified and then the E3 enzyme provides yet another layer of specificity. This is largely true; however, some substrate specificity is determined at the E2 level. E2s can be paired with specific substrates. In yeast for example, Ubc8 has only one known eventual substrate while other E2s are implicated in ubiquitylation of a variety of substrates (Table 1.1). Some E3 enzymes will interact with different substrates depending upon their E2 pair (Madura et al. 1993; Kumar et al., 2010).

1.1.2.6 E2 enzymes and substrate selection

The enzyme UBiquitin protein ligase E3 component n-Recognin 1 (Ubr1), for example, ubiquitylates proteins in two distinct pathways. The way in which Ubr1 functions is determined by the E2s with which it interacts. In yeast, the E2 Ubc2 interacts with Ubr1 to ubiquitylate substrates bearing destabilizing N-terminal residues (Madura et al. 1993). This results in quick degradation. The destabilization of proteins via N-terminal residue recognition and ubiquitylation is known as the N-end rule. (Kumar et al., 2010)

Ubr1 can also function in conjunction with a chaperone protein (Ssa1/2/3/4) in order to degrade unfolded proteins (Nillegoda et al., 2010). The unfolded protein response (UPR) mediated degradation of proteins is an important quality control mechanism. The UPR prevents nonfunctional protein accumulation. In order to fulfill this role, Ubr1 functions primarily with two extremely similar promiscuous E2 enzymes (Ubc4/5) (Sadis et al., 1995). To a lesser extent, Ubr1 can function with two other E2 enzymes (Ubc6/7) in the UPR as well (Stolz, 2011). However, Ubc4/5/6/7 cannot be substituted for Ubc2 with Ubr1 in the N-end rule pathway (Kumar et al., 2007; Kumar et al., 2010; Seufert et al., 1990). If we are to take Ubr1 as an example, it is the E2 that mediates the eventual substrate selection. There is a delicate interplay between E2s and E3s in order to select the eventual substrate of ubiquitylation.

1.1.2.7 E2 enzymes and linkage type specificity

Ubiquitin is typically conjugated to a substrate lysine. This modification can occur at a single amino acid residue (monoubiquitylation) or several residues within the same protein (multi-mono-ubiquitylation). Ubiquitin can also modify itself to form polyubiquitin chains connected to one of the seven lysine residues in ubiquitin. Ubiquitin chains can start with ubiquitin or a non-ubiquitin substrate and are further diversified by the ability to branch (Fushman and Wilkinson, 2011). These different linkage types have different conformations and may be of any length. E2 contributions to substrate selection include selection of linkage type (David et al., 2010). Though E2s are believed to play a role in determining which type of ubiquitylation is to occur, this process and the nature of chain type specificity are currently active areas of research within the field.

Yeast	human ortholog	known function
UBC1	Ube2K	Involved in a variety of processes including the degradation of short-lived and abnormal proteins, ERAD, and vesicle formation.
Ubc2	Ube2A and Ube2B	Functions as a heterodimer in a variety of responces including postreplication repair (with Rad18), N-end rule protein degradation (with Ubr1), and checkpoint control (with Bre1)
Ubc3	Cdc34	works with yeast CRL complexes containing Skp1, Rbx1, Cdc53, and an F-box to ubiquitylate substrates, regulation of cell cycle by regulating levels of important regulators like Sic1, regulation of expression of methionine biosythetic genes
Ubc4	Ube2D family	Very promiscuous. Interacts with many SCF ligases and is involved in polyubiquitylation of many substrates. Has been implicated in the degradation of abnormal or excess proteins, misfolded proteins, and cell stress response. Heavy compensation between Ubc4 and Ubc5 (and to a lesser extent Ubc6 and Ubc7). Expression is heat inducible (part of heat shock response) but typically Ubc4 is expressed in growing cells rather than yeast in stationary phase.
Ubc5	Ube2D family	Very promiscuous. Interacts with many SCF ligases to polyubiquitylate many substrates. Has been implicated in degradation of excess and misfolded proteins as well as functioning in stress response. Expression is induced by heat (part of heat shock response) but typically Ubc5 is expressed in stationary cells rather than growing cells. Heavy compensation between Ubc4/5 (and to a lesser extent Ubc6/7)
Ubc6	Ube2J2	Associated with cytosolic side of ER membrane and involved in ERAD. Some compensation with Ubc7 and Ubc4/5
Ubc7	Ube2G2	Can be recruited to ER and is involved in ERAD. Possible role in chromatin assembly.
Ubc8	Ube2H	Not many known substrates. Degrades fructose-1,6-bisphosphatease to regulate gluconeogenesis. Also reported to ubiquitylate histones in vitro but is a cytoplasmic enzyme and this might not happen in vivo.
Ubc9	Ube2I	Highly specific for functioning as a SUMO E2 enzyme
Ubc10		Peroxisomal E2
Ubc11		Similar to Xenopus enzyme Ube2-C but is not a functional homolog. No known substrates in yeast.
Ubc12	Ube2M/F	NEDD8 specific E2 enzyme
Ubc13	Ube2N	Involved in DNA postreplication repair

Table 1.1. *Yeast E2 enzymes, their human orthologs, and their known functions. Data compiled from Seufert and Jendtsch, 1990, Chen et al., 1993, and the Saccharomyces Genome Database (www.yeastgenome.org).*

1.1.2.8 Degradation within the proteasome

When a protein is tagged with ubiquitin for degradation, that proteinis then shuttled to a macromolecular complex known as the proteasome for processing. Once the protein is delivered to the proteasome, itis denatured and fed through a cylindrical channel at the center of the proteasome. This channel contains 6 distinct proteolytic active sitesthat work sequentially to achieve breakdown of the protein into short peptides. Ubiquitin is released by deubiquitylating enzymes (DUBs) associated with the lid of the proteasome before the substrate is fed into the complex. The released ubiquitin can then be reused. Once the denatured protein is processed, the proteasome isready to accept the next protein.(Chauhan et al., 2005)

1.1.2.9 Ubiquitin stability and turnover

Although ubiquitylation and degradation go hand in hand, ubiquitin itself is stable even under conditions of extreme temperature(Jackson, 2006), pH(Jackson, 2006), and exposure to proteases(Kitahara et al., 2006b). The reportedhalf-lifeof ubiquitin varies between the different tissues, cell lines, and organismsturnover rate has been measured in(Shabek and Ciechanover, 2010). For example, in yeast, the ubiquitin half-life was identified as 2 hours (Hanna et al., 2003)while in HeLa and Chinese hamster ovary cells the half-lifehas been measured atapproximately 9 hours, and about 320 hours in human fibroblasts(Shabek and Ciechanover, 2010).

Ubiquitin turnover has beenreported to be both energy and proteasome dependent. Inhibition of the lysosome only mildly impacted ubiquitin turnover indicating that

degradation of ubiquitin is proceeding through the proteasome.(Shabek and Ciechanover, 2010)

There are several theories on how ubiquitin is degraded. It is possible that in vivo degradation is a combination of all of them. First, ubiquitin can be degraded as a fusion protein with a C-terminal tail of 20 or more residues (Shabek and Ciechanover, 2010). Second ubiquitin can be pulled into the proteasome along with the substrates it is tagging (Shabek and Ciechanover, 2010). This is frequent when the proteasome has a high volume of ubiquitylated substrates to process(Carlson et al., 1987). Alternatively, ubiquitin can be degradedas a monomer that becomes ubiquitylated itself and is degraded by the proteasome. Current evidence indicates that the rate of ubiquitin turnover is independent of conjugation status. There are two reported methods used to study ubiquitin turnover.(Shabek and Ciechanover, 2010)

1.1.2.9.1 Ubiquitin turnover: Pulse chase

A pulse chase system using radiolabeled amino acids has been used to study ubiquitin turnover(Carlson and Rechsteiner, 1987; Shabek and Ciechanover, 2010). This method is useful because it avoids both mutationsand tagging thatmay result in off target effects.

It is possible that this method may not have an entirely accurate readout either. Ubiquitin is a modifier that binds various proteins. Modifiers may have different stabilities depending modification status or the protein that is modified. Ubiquitin cycling on and off of substrates complicates analysis as apparent variations in the ubiquitin level could be due to conjugation rather than degradation. If one were to judge

free ubiquitin levels as a general readout of ubiquitin stability,problems arise when radiolabeled ubiquitin binds substrates. In this case, the free pool of ubiquitin would display reduced labeling but this would not indicate degradation. Furthermore, while ubiquitin conjugated to specific proteins like histone H2A (Chin et al., 1982) have been studied with the pulse chase assay, ubiquitin modified proteins may have substrate specific half-lives so the stability of ubiquitin bound to histone H2A may not serve as an indication of the stability of ubiquitin bound to an unrelated protein. Free ubiquitin may also be more or less stable than ubiquitin conjugated to substrates. In order to minimize these concerns, comparisons have been made between nonconjugateable and conjugateable ubiquitin. Experimental results suggest that ubiquitin turnover is independent of conjugation(Shabek and Ciechanover, 2010).

1.1.2.9.2 Ubiquitin turnover: Diglycine mutation

The second method used to study ubiquitin turnover resolves some of these problems. By preventing conjugation to substrates,the behavior of the ubiquitin monomer could be easily and clearly followed. Initial attempts to prevent conjugation were made by mutatingthe C-terminal glycine (G76) required for thiol-ester bond formation. Mature ubiquitin bearing a mutation of G76 to a nonfunctional alanine does not abrogate interaction with the conjugation cascade,as the penultimate residue is also a glycine that can be utilized by pathway enzymes. However, the G76A mutation inhibits deubiquitylation(Hodgins et al., 1992). Thus, rather than being used as a tool to study free ubiquitin, this method was reported to be a successful means of identifying endogenous substrates of ubiquitylation such as Histone H2B (Geng and Tansey, 2008).

Multiple groups have successfully generated nonconjugateable ubiquitin by mutating (G75/76V, or G75/76A) or deleting the two terminal glycines (ΔGG)(Shabek and Ciechanover, 2010). Such mutations eliminate the Glycine involved in interactions with the E1, E2, substrates, and some E3 enzymes. The diglycine motif is at the end of a short unstructured region at the very C-terminus of ubiquitin(Pickart and Eddins, 2004) which is not believed to contribute much to structural integrity under non-extreme temperatures (Jackson, 2006). The diglycine mutation methodreducesthe concern that apparent ubiquitin degradation is due to conjugation because it can no longer become conjugated.

1.2 Ubiquitin like Proteins

Ubiquitin like modifiers (UBLs) share some sequence, regulatory, and structural similarity with ubiquitin. These UBLsinclude NEDD8, small ubiquitin-like modifier (SUMO), interferon-stimulated gene-15 (ISG-15), ubiquitin related modifier-1, and Human leukocyte antigen-Fassociated transcript 10(FAT10). (Jentsch and Pyrowolakis, 2000)

1.2.1NEDD8

Neurologically Expressed Developmentally Downregulated 8 (NEDD8), the UBL most like ubiquitin, was identified in 1992 in mouse neural precursor cells (Kumar et al., 1992). In some species, such as baker's yeast, the NEDD8 homolog is known as related to ubiquitin 1 (Rub1). NEDD8 and ubiquitin share the highest degree of homology to ubiquitin of any of the ubiquitin like modifiers;approximately 60% similarity and 80% homology (Figure 1.2). Like ubiquitin, NEDD8 is highly conserved in eukaryotes,

shares a similar backbone topology and is synthesized as an inactive precursor that, once processed, isable to function as a posttranslational modifier. These UBLs share similar conjugation cascades dependent upon the activity of E1, E2, and E3 enzymes to conjugate their C-terminal diglycine motif to a substrate lysine. The bulky Arg 72 in ubiquitin (Ala in NEDD8) acts as a selectivity gate to insulate the neddylation pathway from consuming ubiquitin(Souphron et al., 2008).

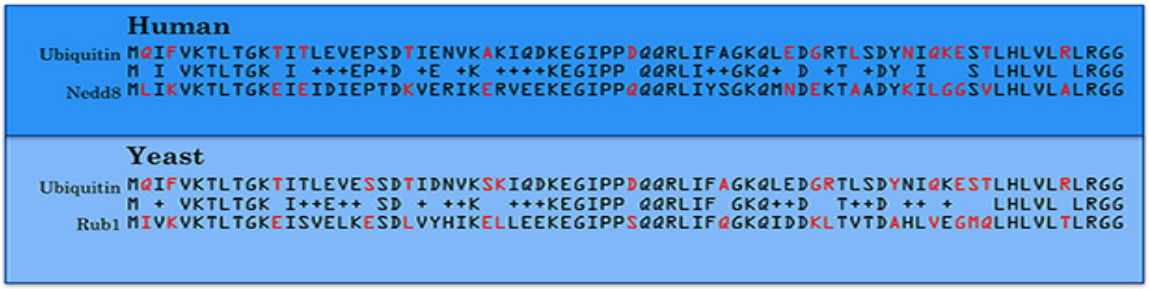


Figure 1.2. Comparison between human and yeast ubiquitin and NEDD8/Rub1 sequences. Residues which are found to be significantly different are highlighted in red. Similar residues are indicated by the + sign.

1.2.1.1 Importance of NEDD8

NEDD8 is essential for viability in all organisms tested with the exception of *Saccharomyces cerevisiae* (yeast). In yeast the NEDD8 homolog, Rub1 functions much like NEDD8 but is nonessential. While the loss of Rub1 results in some phenotypic changes, yeast lacking the *rub1* gene is still viable and grows well under standard conditions.(Finley et al., 2012)

1.2.1.2 Ubiquitin and NEDD8 maturation

In order to become conjugated and deconjugated from substrates, ubiquitin and NEDD8 typically follow parallel but demonstrably unique conjugation cascades (Figure 1.1). They are both synthesized as precursors that need to have their C-terminal extensions

cleaved to reveal a diglycine motif (G75/76). This maturation is believed to be accomplished primarily through the proteolytic activity of ubiquitin C-terminal hydrolase (UCH) domain containing deubiquitylating enzymes. Interestingly UCHL3, the DUB primarily responsible for maturation of NEDD8 has dual specificity for both ubiquitin and NEDD8. (Watson et al., 2011)

1.2.1.3 Ubiquitin and NEDD8 conjugation cascade

Following maturation, ubiquitin and NEDD8are activated by an E1 activating enzyme (UAE and NAE, respectively). In the case of NEDD8/Rub1 the NAE is a heterodimer composed of UBA3 and APPBP1 (Uba3 and Ula1 in yeast). The UBLs are subsequently transferred from the active site cysteine of the E1 to the active site cysteine of an E2 conjugating enzyme. There are many E2s that can accept ubiquitin from the UAE but only UBC12 and UBE2F can accept NEDD8 from the NAE in mammals (only Ubc12 in yeast) (Gong and Yeh, 1999; Huang et al., 2009; Wada et al., 2000). Lastly, through the action of an E3 ligase,the UBLs become conjugated to substrates (Figure 1.1). The terminal glycine of the UBL is bound to the substrate most characteristically through an isopeptide bond. As with ubiquitylation, neddylation occurs on a substrate lysine. While ubiquitin has a great deal of diversity in available E3 enzymes, in the neddylation pathway,defective in cullin neddylation (DCN) proteins coordinate witha RING domain protein(RBX1 or 2) to facilitate substrate neddylation(Finley et al., 2012; Watson et al., 2011).Other E3 enzymes have been reported, such as MDM2 (Watson et al., 2006), but these enzymes have not been shown to be dependent upon the NAE.

1.2.1.4 Ubiquitin and NEDD8 crosstalk

Under endogenous unstressed conditions crosstalk between the NEDD8 and ubiquitin pathways has not been reported. However, overexpressing NEDD8 or inducing cellular stress responses can perturb the ratio of ubiquitin to NEDD8and some crosstalk can occur(Hjerpe et al., 2012a; Hjerpe et al., 2012b; Leidecker et al., 2012; Whitby et al., 1998). While ubiquitin is not activated by the NAE (Souphron et al., 2008), activation of NEDD8 by the UAE is observed in vivo whenNEDD8 levels are elevated or ubiquitin levels are depleted(Hjerpe et al., 2012a; Hjerpe et al., 2012b; Leidecker et al., 2012; Whitby et al., 1998). Once NEDD8 is conjugated to the UAE, it can proceed via the ubiquitylation machinery. One of the great pioneers in the field, Cecil Pickart demonstrated in vitro that misloading of E2-25k with NEDD8occurred with about the same efficiency as loading with ubiquitin(Whitby et al., 1998). Relatedly, we recently published that numerous E2s can be loaded with NEDD8(Hjerpe et al., 2012a). Once NEDD8 is activated by the UAE, it can proceed relatively unhindered through most ubiquitin E2 enzymes and ultimately become conjugated to substrates that are normally ubiquitylated such as Hif1α(Hjerpe et al., 2012b).

1.2.1.5 Substrates of neddylation

While there are numerous endogenously identified substrates of ubiquitylation, there are comparatively few neddylated proteins identified to date. The most well-known substrate of neddylation is the cullin family of proteins(Watson et al., 2011). Neddylation of the cullins epitomizes the interplay between neddylation and ubiquitylation because cullins are a key component of the largest class of E3 ubiquitin ligase.

1.2.1.5.1 Non-cullin NEDD8 substrates

Reports of neddylation of non-cullin proteins rely heavily upon overexpression. NEDD8 substrates identified by NEDD8 overexpression include proteins like AICD, XIAP, and p53 (see Table 1.2 for a list of non-cullin neddylated proteins). Recent articles elucidating the accepted means of identifying neddylated proteins still rely upon overexpression (Leidecker and Xirodimas, 2012). However, as we have reported, overexpression can shunt NEDD8 onto the ubiquitin pathway(Hjerpe et al., 2012a). There is growing evidence that many of these reported substrates proteins are neddylated in a UAE rather than a NAE dependent manner (Table 1.2). Neddylation of p53, for example, is heavily dependent upon the UAE (UBE1) rather than the neddylation pathway (Hjerpe et al., 2012b).

Some of the reported non-cullin NEDD8 substrates could be neddylated endogenously. Gao et al. demonstrated that co-overexpression of BCA3 and p65 is sufficient for neddylation of BCA3 even without NEDD8 overexpression. Under such conditions, the risk of NEDD8 misactivation is minimized and the described interaction may be real. Inhibition of the NAE could confirm this interaction. The neddylation of another putative substrate, p73, has mostly been studied using overexpression but, Watson et al. also immunoprecipitates endogenous p73 and finds weak modification by NEDD8. BCA3 and p73 may be endogenously neddylated. While other reported non-cullin proteins may be endogenously neddylated, this has not been conclusively demonstrated (Table 1.2).

Protein	Neddylation Reference	Rebuttal Reference

AICD	(Lee et al., 2008)	
BCA3	(Gao et al., 2006)	
Caspase 7	(Broemer et al., 2010)	(Hjerpe et al., 2012b) (Nagano et al., 2012)
EGFR	(Oved et al., 2006)	
HIF1α	(Ryu et al., 2011)	(Hjerpe et al., 2012b)
HIF2α	(Ryu et al., 2011)	
MDM2	(Xirodimas et al., 2004) (Watson et al., 2010)	
p53	(Abida et al., 2007) (Xirodimas et al., 2004) (Leidecker and Xirodimas, 2012)	(Hjerpe et al., 2012b)
p73	(Watson et al., 2006)	
pVHL	(Russell and Ohh, 2008) (Stickle et al., 2004)	
Ribosomal proteins (ex. L11)	(Xirodimas et al., 2008)	
XIAP	(Nagano et al., 2012)	

Table 1.2. *Reportedly neddylated non-cullin proteins. Proteins besides the cullins which are reportedly neddylated are listed by sources supporting or denying their neddylation status.*

1.2.1.5.2 Cullins

The cullin family contains the most robustly supported endogenously neddylated proteins identified to date.

1.2.1.5.2.1 Cullin neddylation

The cullins all share a conserved C-terminal neddylation site downstream of a conserved cullin homology domain. With the exception of cullin 7 the cullins have been shown to be neddylated. Although the neddylation site and cullin homology domain are conserved in cullin 7, it is unclear whether this cullin is endogenously neddylated.(Sarikas et al., 2011)

1.2.1.5.2.2 Cullin function

Cullin RING Ligases (CRLs), the main class of E3 ubiquitin ligases, facilitate the movement of ubiquitin from E2 to substrate by acting as a scaffold for the assembly of the whole E3 complex. Substrates are linked to the N-terminal region of the cullin via an adaptor and a substrate recognition protein. At the C-terminal region of the cullin, a RING finger protein (Rbx1/2) joins the cullin to the ubiquitin charged E2 enzyme. Neddylation at a conserved residue of the C-terminal domain of the cullin allows for the C-terminal flexibility required to bridge a 50Å gap between the substrate and the ubiquitin (Figure 1.3 adapted from (Duda et al., 2008)).

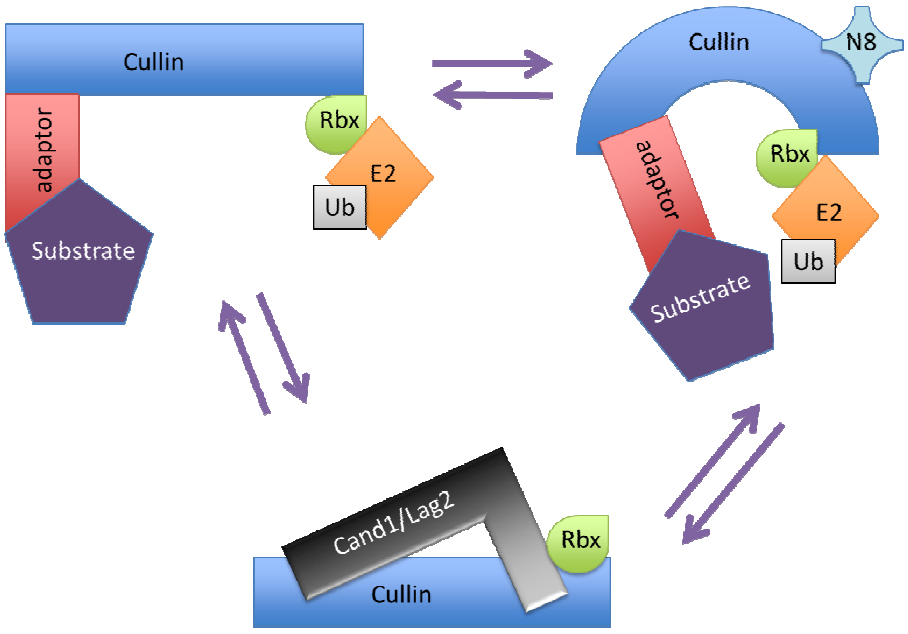


Figure 1.3. Schematic of the regulation of CRL complexes by NEDD8 and CAND1/Lag2. N8 denotes NEDD8, Ub denotes ubiquitin, Rbx denotes the RING proteins from the Rbx family.

1.2.1.5.2.3 Regulation of cullin neddylation

Cullin associated neddylation dissociated1 (CAND1, Lag2 in yeast) binds to cullins such as Cul1, 2, 3, 4A, and 4B preventing conjugation of NEDD8 (Figure 1.3) (Chua et

al., 2011; Zheng et al., 2002). There are conflicting reports on the importance of CAND1 binding to CRL function. There are some groups that have used proteomic approaches to argue that cycles of neddylation and deneddylation are not significant in overall CRL function(Bennett et al., 2010). Others report that CAND1 is an important regulator of the CRL network(Goldenberg et al., 2004; Sela et al., 2012). Recent work by Schmidt et al., 2009, andwithin our lab, primarily by Aleksandra Zemla,indicate that the role CAND1 plays may be specific to certain CRLs by regulating substrate adaptor exchange. Such behavior would explain these divergent observations.

1.3 Deubiquitylases and deneddylases

One of the key elements of the posttranslational UBL modification scheme is that it is reversible. UBLs may be removed from substrates through cleavage of the isopeptide bond by DUBs. There are six families of DUB each bearing unique defining characteristics (Table 1.3 adapted from Reyes-Turcu et al., 2009). Some DUBshave a high degree of specificity for a specific UBL or for leaving group size while others are fairly promiscuous. For example, NEDD8 specific protease 1 (NEDP1) has a 60,000 fold higher specificity for NEDD8 than for ubiquitin(Gan-Erdene et al., 2003)but ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCHL3) has dual specificity for NEDD8 and ubiquitin(Frickel et al., 2007).

1.3.1 Deneddylases

Most DUBs do not process NEDD8 at all. The only reported mammalian deneddylases to date are NEDP1(Gan-Erdene et al., 2003), CSN5(Cope et al., 2002), UCHL3(Frickel et al., 2007), USP21(Gong et al., 2000), UCHL1(Hemelaar et al., 2004), and Ataxin-3 (Ferro et al., 2007). Of these 6 putative deneddylases, the evidence for the first 3 is strong while the evidence for the other 3 is relatively weak.

Family	Alias	type of protease	Notable Characteristics
UCH	Ubiquitin C-terminal hydrolase domain	Papain-like Cysteine proteases	Preferentially cleave small leaving groups
UBP/ USP	Ubiquitin specific protease domain	Papain-like Cysteine proteases	Preferentially cleave larger leaving groups. Largest family (56/85 DUBS in humans, 16/19 DUBS in yeast), very diverse with low sequence similarity but high conservation in the USP domain fold
OTU	Ovarian tumour domain	Papain-like Cysteine proteases	Not all OTU domain proteins have DUB activity
MJD	Machado-Josephin domain	Papain-like Cysteine proteases	Structure similar to the UCH domain
JAMM	Jab1/MPN/ Mov34 Metalloenzyme domain	zinc metalloproteases	NEDD8 and K63-linked ubiquitin
ULP	Ubiquitin like protease domain	Adenain family of cysteine proteases	Specificity for UBLsrather than ubiquitin. Some debate as to whether it should be listed as a DUB

Table 1.3. *Families of DUBs. Distinguished by protease type and notable characteristics as adapted from Reyes-Turcu et al., 2009.*

1.3.2 Commonly accepted deneddylases

1.3.2.1 NEDP1

NEDP1 is a cysteine protease of the ULP family that can act as a deneddylase in fission yeast, plants, and animals but is not present in *S. cerevisiae*(Mendoza et al., 2003). Though no endogenous NEDP1 substrate has been identified, it has been shown in a variety of overexpression and in vitro experiments to cleave NEDD8 from many substrates. NEDP1 displays very pronounced specificity for NEDD8 over ubiquitin but is fairly promiscuous with respect to the protein to which NEDD8 is conjugated.

Little work has been published about NEDP1 at the endogenous level. One possible endogenous substrate is breast cancer-associated protein 3 (BCA3). BCA3 was identified as a NEDP1 interaction partner by yeast-2-hybrid screening. While most of the work done relating these two proteins involved overexpression (and therefore could be the result of misactivationof NEDD8or deneddylation of the cullins), there is some evidence of genuine BCA3/NEDP1 interaction. For example, knocking down NEDP1expression by siRNA results in an increase in a higher molecular weight form of endogenous BCA3 in the nuclear fraction of HeLa cells(Gao et al., 2006). Furthermore when BCA3 is overexpressed, and immunoprecipitated, a NEDD8 modified form is visible even when NEDD8 is not overexpressed(Gao et al., 2006). Such evidence could indicate that BCA3 may be a genuine substrate of NEDP1.

1.3.2.2 CSN 5

CSN5(Rri1 in yeast) is a subunit of the mutlimeric COP9 signallosome containing a JAB1/JAMM metalloenzyme domain responsible for deneddylation of the cullins. CSN5 has a well established role in cullin deneddylation.There are no other reported substrates deneddylated by CSN5. (Wei and Deng, 2003)

1.3.2.3 UCHL3

UCHL3 (Yuh1 in yeast) is unique in that it is the only one of the well-established deneddylases to cross react with ubiquitin. It has been shown that UCHL3 protects the ubiquitin dimer(Setsuie et al., 2009a) and plays a role in ubiquitin maturation. UCHL3 even cleaves the 19-residue extension on a misread ubiquitin product known as UBB+1(Dennissen et al., 2011). The accumulation of the uncleaved form is found in AD affected regions of the brain and is transiently upregulated in affected areas of the brain following a stroke. There is also compelling evidence demonstrating that UCHL3 matures pro-NEDD8. UCHL3 is also able to interact with a NEDD8 reactive species at the right molecular weight for a NEDD8 dimer (Setsui et al., 2009a) but this species has not been conclusively identified.

Of the three well-established deneddylases, UCHL3 has been most extensively studied. Misregulation of UCHL3is connected to cancer(Miyoshi et al., 2006). UCHL3 knockout mice have tissue specific phenotypes incuding short term memory loss(Wood et al., 2005), enlarged mitochondria and oxidative stress apoptosis in the photoreceptors of the retina(Sano et al., 2006), and susceptibility to heat stress in testicular germ cells(Kwon et al., 2004). In skeletal muscle the knockout mice also display accumulation of polyubiquitylated proteins, activation of the unfolded protein response,

heat shock response, and degeneration(Setsuie et al., 2010). These mice also presented with resistance to diet induced obesity as well as a reduction in visceral fat and increased fatty acid oxidation in skeletal muscles (Setsuie et al., 2009b). It is unclear whether these phenotypes are a result of the failure of UCHL3 as a deneddylase, a deubiquitylase, or both.

1.3.2.4 Reported deneddylases with questionable activity

While USP21 and UCHL1 have demonstrable deubiquitylase activity, there are conflicting reports on whether they also have deneddylase activity. The most recent data suggests that they are not NEDD8 reactive(Wada et al., 1998; Ye et al., 2011). The data suggesting that Ataxin-3 has deneddylase activity was reported from one source alone (Ferro et al.2007) using in vitro and overexpression conditions which can both result in misregulation of NEDD8.

1.4 Regulation of NEDD8

NEDD8ultimate buster 1 (NUB1) was originally reported as a regulator of NEDD8 stability. As reported by Hipp et al. in 2004,the NUB1/NEDD8 interaction is largely tag dependent and NUB1 is actually regulating the turnover of the UBL FAT10. NEDD8 turnover is reportedly rapid (half-life of 3.5 hours in HEK293 cells) and not highly regulated by NUB1. Recent publications reinforce acceptance of the observation that NEDD8 turnover is rapid (Buchsbaum et al., 2012) but the mechanism of turnover has not been elucidated.

1.5 Inhibition of Neddylation

Defects in the regulation of cullin neddylation can result in cell cycle defects (Tateishi et al., 2001). This trait is being exploited in the development of a new anti-cancer agent known as MLN4924 (Nawrocki et al., 2012). The functional success of MLN4924 as a drug may be related to inhibition of cullin neddylation, but if there are other NAE dependent substrates of neddylation these too would be affected by treatment with MLN4925 because NEDD8 cannot procede beyond the NEDD8 E1.

1.6 Aims

The primary aim of this work is to understand NEDD8 specific regulatory mechanisms. We are primarily focused on the basic biological question of how NEDD8 is regulated to prevent cross-talk with the ubiquitin pathway. We have parsed this question into two parts. First, how is free NEDD8 regulated to prevent alterationsin the ratio of NEDD8 to ubiquitin that may result in misactivation by the UAE. Second, which substrates are endogenously neddylated in a NAE dependent manner. Beyond a basic question of biology, this project has health implications as well. The targeting of the neddylation machinery as an anti-cancer treatment may impact pathways that are not well defined. Until we know which proteins are genuinely neddylated we will be unsure what MLN4924 does to patients at a molecular level.

In order to address these concerns we used yeast to examine Rub1 turnover and conjugation but were unable to identify the endogenous degradation pathway or non-cullin substrates. We also attempted to identify novel substrates of neddylation in cultured cells and mouse tissues. We were unable to clearly identify substrates of neddylation beyond the cullin family. However we were able to determine that

neddylation is upregulated in testes. We were also able to identify certain families of proteins coimmunoprecipitating with NEDD8 in testes including coatomer, matrin-3 associated proteins, and structural maintainence of chromosome proteins. Other members of the NEDD8 associated proteome include CAND1 as well as cullin 7 (the only cullin not shown to be neddylated (Skaar et al., 2007; Sarikas et al., 2011)). Overall, we were unable to identify novel regulatory mechanisms or substrates of neddylation.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Commercial reagents

- Amicon Ultra "ultracel 5K" (Millipore)
- 0.05% Trypsin-EDTA (Invitrogen)
- 0.22 µm sterile syringe filters (Sartorius Stedium)
- 1kb DNA ladder (New England Bio Labs)
- 20X MES Running buffer (Invitrogen)
- 20x MOPS Running buffer (Invitrogen and Formadium)
- 20X Transfer buffer (Invitrogen)
- 3mm chromatography paper (Whatman)
- 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) HEPES (BDH)
- 6-Well plates and T75 flasks (Corning)
- 6x DNA loading dye (Promega)
- Acetic acid (BDH)
- Acetone (BDH)
- Acetonitrile (ACN) (Millipore)
- Acid-washed glass beads (Sigma)
- Acrylamide (Flowgen Biosciences)
- AminoLink Plus Immobilization Kit (Pierce)
- Ammonium bicarbonate (ABC) (Fluka)
- Ammonium persulfate (BDH)
- ATX Ponceau S red staining solution (Fluka analytical)

- BCA Protein Assay Kit (Pierce)
- BD granulated agar (Merck)
- Beta-mercaptoethanol (Calbiochem)
- Biophenol (BDH)
- Bis-Tris NuPAGE Gels (Invitrogen)
- Bovine serum albumin (Roche)
- Bromophenol Blue (Merck)
- Calcium chloride (BDH)
- Carbenicillin (amp) (Formedium)
- Centrifuge tubes for TL-100 Ultracentrifuge (Beckman)
- Chemiluminescence X-Ray films (GE Healthcare)
- ClonNat (Werner BioAgents)
- Cobalt (II) chloride (Sigma)
- Complete EDTA-free Protease Inhibitor Cocktail (Roche)
- Control agarose resin (Sigma)
- Coomassie protein assay reagent (Thermo)
- Crosslink immunoprecipitation kit (DSS crosslinking) (Pierce)
- Cuvettes (Starstedt)
- Dehydrated culture media (agar granules for yeast plates) (BD Difco)
- Dithiothreitol (DTT) (Formedium)
- DMSO (BDH)
- Dried skimmed milk (Marvel, Premier Beverages)
- Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen)
- EDTA (BDH)

- Enhanced Chemiluminescent reagent (ECL) (Millipore)
- Ethanol (BDH)
- Ethidium Bromide (BDH)
- Glycerol (BDH)
- Glycine (BDH)
- High Fidelity Polymerase Kit (Roche)
- His drop out mix (Formedium)
- Human CSN5 ON-TARGET plus SMARTpool siRNA (Dharmacon)
- Human Senp8 ON-TARGET plus SMARTpool siRNA (Dharmacon)
- Human Senp8 siRNA (Ambion)
- Human UCHL3 ON-TARGET plus SMARTpool siRNA (Dharmacon)
- Innoculating Loops (Thermo)
- Instant Blue (Expedeon)
- Iodoacetamide (Sigma)
- Isopropanol (BDH)
- Kanamycin (Formedium)
- L-glutamine (Invitrogen)
- Lipofectamine RNAiMAX for siRNA knock-down (Invitrogen)
- Lithium acetate (Alpha Aesar)
- Magnesium Chloride (Sigma)
- Magnesium sulfate (BDH)
- MG132 (Enzo)
- Microspin C18 silica column (Nest group)
- Monoclonal anti-HA agarose conjugate (Sigma)

- N,N,N',N'-Tetramethylethylenediamine (TEMED) (BDH)
- NHS-activated M-PV magnetic beads (Perkin Elmer)
- Nitrocellulose membrane (GE Healthcare)
- Novex tricine gels for small protein separation (Invitrogen)
- Novex tricine SDS loading buffer (Invitrogen)
- Novex tricine SDS running buffer (Invitrogen)
- OptiMEM (Invitrogen)
- PCR Purification Kit and Plasmid DNA Minprep kit (Qiagen)
- PCR Tubes (Thermo)
- Penicillin and Streptomycin (Invitrogen)
- PhosSTOP Phosphatase Inhibitor cocktail (Roche)
- Photographic Fixer and Developer Solutions (Redichem)
- Polyethylene glycol 3550 (BDH)
- Polyethylene glycol 4000 (BDH)
- Potassium acetate (BDH)
- Potassium chloride (BDH)
- Potassium hexacyanoferrate (III) (Sigma)
- Protein G agarose (Pierce)
- QIAprep Spin Miniprep Kit (Qiagen)
- Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX (Invitrogen)
- Salmon Sperm DNA (Sigma)
- See Blue Plus 2 protein ladder (Invitrogen)
- Sequencing grade modified trypsin (Promega)
- Silver Stain for Mass Spectrometry (Pierce)

- Sodium bicarbonate (BDH)
- Sodium chloride (BDH)
- Sodium Dodecyl Sulfate (BDH)
- Sodium hydroxide (BDH)
- Sodium thiosulfate (Sigma)
- Sterile filter units (Millipore)
- Trichloroacetic acid (BDH)
- Triethyl ammonium bicarbonate (TEABC) (Sigma)
- Trifluoroacetic acid (TFA) (Thermo)
- TRIS(hydroxymethyl)aminomethane (BDH)
- Tween-20 (BDH)
- UltraPure Agarose (Invitrogen)
- Ura drop out mix (Formedium)
- Urea (BDH)
- Yeast extract (Merck)

2.1.1.1 Commercial antibodies

The commercial antibodies used in this project are listed below in Table 2.1.

Antibody Name	Company	Product number	Source
Anti-Actin	Millipore	mab1501R	Mouse
Anti-GFP	Roche	11814460001	Mouse
Anti-HA (HA.11)	Covance	MMS-101R	Mouse
Anti-HA-HRP	Sigma	H6533	Mouse
Anti-NEDD8 (Epitomics)	Epitomics	1571-1	Rabbit
Anti-NEDD8 (Mil10)	Millenium Pharmaceuticals	N/A	Rabbit
Anti-protein G-HRP	Abcam	AB7250	Rabbit
Anti-Tubulin	Sigma	T9026	Mouse
Anti-ubiquitin	Dako	Z0458	Rabbit
Anti-UCHL3	Cell Signalling	3525	Rabbit
Mouse-HRP	Biorad	170-5047	Goat
Rabbit-HRP	Biorad	170-5046	Goat
Sheep-HRP	Abcam	ab97130	Rabbit

Table 2.1.*Commercially produced antibodies used in this project. Mil10 was a gift from Millennium Pharmaceuticals and is not available for sale but all others were purchased.*

2.1.1.2 Instruments

- Automatic film processor SRX-101A (Konica Minolta)
- Avanti J-25 Beckman
- Balances: PL300-S (Mettler Toledo), LP2200P (Sartorius), Acculab
- Centrifuge 581R Eppendorf
- Centrifuge Beckman Coulter J6-Mi
- Centrifuge Beckman TL-100 Ultracentrifuge
- DNA Engine Peltier Thermal Cycler (BioRad)
- EnVision 2104 Multilabel reader (Perkin Elmer)
- Epson Perfection V700 PHOTO scanner (Hertfordshire, UK)

- Freezer Mill 6770 Spex Sample Prep
- Heat block (Eppendorf)
- Infors HT Multitron Incubators
- Microscope Primo Star (Zeiss)
- Milli-Q Biocel system (Millipore) with a Quantum EX Ultrapure Organex cartridge (Millipore)
- Mini-SubCell GT (BioRad)
- Molecular Imager Gel Doc XR+ Imaging System (BioRad)
- Multichanel pipettes (Star Lab)
- NanoVue version 4282.V2.0.3 (Nanodrop) (GE Healthcare)
- Odyssey Infrared Imaging Systim (Li-COR)
- pH meter (Hanna Instruments)
- Pipette-Aid (Gilson)
- Power-Pac Basic Power Supply (BioRad)
- Rotator Drive (wheel) STR4 (Stuart)
- Savant SPD131 DDA SPeedVac Concentrator (Thermo)
- See-saw rocker SSL4 (Stuart)
- Solution mixer/heater CB162 (Stuart)
- Sonicator (Bandelin Electronic)
- Spectrophotomeer Ultraspc 2100 pro (Amersham Biosciences)
- Static Incubators (Binder)
- Table top centrifuges: Heraeus Pico 17 and Heraeus Fresco 17 (Thermo)
- Tissue culture CO_2 Incubators (Binder)
- Tissue culture lab microscope (Leica)

- Tissue culture safety cabinet BioMAT-2-SF (Medical Air Technology)
- Vortex-Genie (Scientific Industries)
- X-Cell Sure Lock Mini-Cell Electrophoresis system and X-Cell II Blot Module (Invitrogen)

2.1.2 In house reagents

Both liquid media and agar plates for yeast and bacteria culture were prepared by the Kitchen Service, University of Dundee. The only difference between the recipes for liquid media or agar plates was the omission of agar from the liquid media.

Luria Bertani (LB) broth
1% tryptone
0.5% yeast extract
1% sodium chloride
2% bacto-agar
YPAD
1% bacto-yeast extract
2% bacto-peptodne
2% glucose
0.004% adenine sulfate
2% bacto-agar
Synthetic Dextrose Minimal Media (SD)
0.67% bacto-yeast nitrogen base without amino acids
2% glucose
2% bacto-agar
Supplemened with appropriate amino acids or a drop-out mix for plasmid selection

Table 2.2. *Media recipes used to generate growth media for bacteria and yeast.*

10X TBS and PBS were also prepared by the Kitchen Service. MLN4924 was synthesized by Natalia Shapiro at the University of Dundee.

2.1.2.1 Primers

Oligonucleotide primers for yeast DNA were designed using the sequences available on the *Saccharomyces* Genome Database (www.yeastgenome.org) and DNA Strider 1.4 for OS X. Primers were synthesized by the University of Dundee Oligonucleotide Synthesis Service upon request and are listed below in Table 2.3.

Identification number	Target gene	Oligonucleotide location (bp)	Sequence
oSC10	UBC12	268 upstream	CTGCCTTAAACGAGTGGCG
oSC11	UBC13	253 upstream	GCCTTCTCTACATGTGAAC
oSC12	UBC4	251 upstream	GTACCGGCGGTCACATGG
oSC13	UBC5	251 upstream	GATGTATTGCTAGTGCTAG
oSC14	UBC8	~250 upstream	GCTCGTGTGCACATCTGCG
oSC18	UBC1	274 downstream	CCGCAGTCGGTCCCCTGATCGC
oSC29	UBA3	464 upstream	CTCAAGCAACACTGAGGTCAC
oSC30	UBA3	359 downstream, reverse	CGTGACATCCACCACTTTC
oSC6	UBC10	255 upstream	CGCGTTACCCGTATCATC
oSC7	UBC7	276 upstream	GCACACGCATATTTGTTCCC
oSC8	UBC2	277 upstream	GTGTGAGCTAACCATGCT
oSC9	UBC11	253 upstream	CAATAGTCCTGCATACGTAGC
oTK82	RUB1	Downstream	TATCGTAATCGCATTTTACATAAGG
oTK83	RUB1	Upstream	AATCAGACCATATATAGCC
oTK98	KAN	Center reverse Kan casette	GATGGTCGGAAGAGGCATAA

Table 2.3. *Primers used in this project. All primers were generated in house. The oligonucleotides designed by myself are denoted with an oSC number while the three oligomers designed by Thimo Kurz are denoted by oTK numbers.*

2.1.2.2 Plasmids

All plasmids, cloning, subcloning, and mutagenesis was performed by Nicola Wood, Zoey Gage, Thomas Macartney and Melanie Wightman of the MRC/SCILLS Cloning Service, University of Dundee, upon request using the QuickChange site directed mutagenesis method (Stratagene # 200518). The resultant PCR product was subcloned into pYES2 or pRS413 vectors. For galactose inducible overexpression constructs, the

pYES2 vector was used while the centromeric pRS413 vector was used for a lower level of constitutive expression. The backbones both carry ampicillin resistance, which was used to select for bacterial transformants. Sequencing was confirmed by the University of Dundee DNA Sequencing Service using DYEnamic ET terminator chemistry (Amersham Biosciences) on automated DNA sequencers (Applied Biosystems). The plasmids were subsequently maintained and amplified in competent DH5α *E. coli*.

Vector	5' UTR	Expressed protein	3' UTR	Cloning Service	T. Kurz
pYES2		3HA-Rub1 ΔGG		x	
pYES2		3HA-GFP-8 x Gly-Rub1		x	
pYES2		3HA-GFP-8 x Gly-Rub1G75/76A		x	
pYES2		HA-Rub1 G75/76A		x	
pYES2		Rub1		x	
pYES2		Rub1 G75/76A		x	
pRS413	x	Rub1		x	
pRS413	x	Rub1	x	x	
pRS413	x	Rub1 G75/76A	x	x	
pRS413	x	HA-Rub1 G75A	x	x	
pRS413	x	HA-Rub1 G75/76A	x	x	
pRS413	x	HA-Rub1 ΔN77	x	x	
pRS413	x	HA-Rub1 G75A ΔN77	x	x	
pRS413	x	HA-Rub1 G75/76A ΔN77	x	x	
pRD54	x	3HA-6 x HIS-Rub1			x
pRD54	x	3HA-6 x HIS-Rub1 ΔGG			x

Table 2.4. *Plasmids used in this project. The plasmids are identified by backbone, inserted sequence and source.*

The 5'UTR (Rub1 promoter) was defined by the following sequence:

CTCGAGCTCAAAAAAGATATTCTGTCATGAGCATAGCTATGAATTTTTCT

TTATAAACCGAAATATAGTTATACGCTTTGAGTAAAGACACAGGTTATCA

ATTATAACGTGAAAGGTTTTGCAACTGTCACCTTGTAAAGCTGTACACAC

TGAGGACTTCCCATCCCGCTTTTCTCGTTTCAATAAGTTTCACATTCCAT

TTGTAAGTATCGACAAACCTCTTAATGACTTCTTTAGGCTTATTTCTTGC

ACAAAATAATATATATGCAACACCATCTGGAGAAAGAATTTGTTCTAATT

GTCGTAGCAGTTTATCAGTAATCGCCATCCCGTCTTTTCCACCCAATAAC

GCTAAATCTAGCCATTGGTCTGCCTCCTCTCTTGATCCTGGGACATCTGG

CACACATTCTGCTGGCACATATGGTGGGTTAAATATTAGGACATCAACCT

GATTATTCCGAATACTGGAGTTTAAATCAGCTTGAATCACTTCTAAAAAA

GAACTTTTACATGAATTTAACTTCGCAGTATCCAAAGTTGCTTCGAGCGC

CCATGGGTTGATATCAACAGCTAAGTGGATGGAATTTTCCTGCGGTATTA

TTTTGTTTTGCATTAGAAATGTTGTGACGATACCTGATCCCGAACCAATT

TCGCAAACGATGGCCAAACGATTACCAAATTTCTGTTTCAAAAAATCATG

CTCTTTTTCCAAACAGTCCAGTATGAGGAAGCTATCCTCAGCAGGCTCAT

ATACTTTATCGTAATCGCATTTTACATAAGGGGTCGGTAGCATACTTCAC

CTTTCTCACCCTGTGTTAAGTTTATCAATATTATATTTCAGAGTATAATT

ATATCAATATTTATATGAAATCAAATTCCGATGGCGTCTTCGGGAAGGCA

AACGAACAACTTTTAGCTGAAAAACCAAAATTCTGTTATTCAAATGAAGT

ATTCCGACAGAGGAATAAATAAAGGAAGGTAATTAACTTCCTTACAGCCG

TAACCGGAATTC

The 3’UTR was defined by the following sequence:

AACCATTTTCCGTCATTTATGGAATTTTATCGAAGGTGTTCATTAGAAAAGA

AGACAAAACGAACATAAGTTCACTTGGACTTTGGAAGGCTACCGCCCCCAA

TTTGTCAAAAAAAAAAATACATACAATTTAGTCTATAGAGTCTGTTATAATA

AATTAATTGTTTTTAGACGAGAAATGTGAACTAAAACTGTTTAGTATCTATC

AAATTATCCGTTATAGCATAGTACGGCTATATATGGTCTGATTGCTGGTCTC

ATATGACTTTATGGTTTAAATTTCATAAAGCTGGCCATTGCATGGTTCGCGC

TGGAAAGAGAGGAAAAGCGTGGTGATAAAGAGCAAACCAAGCATACTGTT

ATATGCGATTACAGACTATCTGACCAAAAGACATATTTCGCCATTTCTTCTC

AATCATGTCTTCCAGGGCAATTTTGTAAGCACGATTGGATGTCCTCTTGTAG

TATACCGAATTTCTCATTAAGAAAAGCATCCTTTGCGCGGCCGC

2.1.2.3 Proteins

The following proteins were obtained from the Scottish Institute for Cell Signalling Protein Purification and Assay Development Team (PPAD), led by Dr. Axel Knebel at the University of Dundee:

> GST, GST-NEDP1 C163A, His-HA-NEDP1 C163A, NEDD8, Rub1, yeast ubiquitin, and human ubiquitin

PPAD expressed these proteins in bacteria and purified them from inclusion bodies using ion exchange and size exclusion chromatography. Proteins were then aliquoted and stored in Ubiquigent Buffer (50mM HEPES pH 7.5, 10% glycerol, 150mM NaCl, 1mM DTT) at -80°C and thawed on ice as needed.

2.1.2.4 In house antibodies

There were a series of antibodies produced in-house by the Division of Signal Transduction Therapy (DSTT), University of Dundee, utilized in this project. Additionally, pre-immune IgG was purified by the DSTT from non-immunized sheep serum using a protein G-sepharose column. All antibodies were affinity purified on CH-sepharose covalently coupled to the appropriate antigen. These antibodies are as listed below in Table 2.5. Additionally, the anti-NEDP1 antibody was developed for

the laboratory of Dr. Dimitri Xirodimas and an aliquot was generously donated to the Kurz laboratory to validate NEDP1 knockdown experiment.

Name	Animal	Number	Immunogen Sequence	For this project
CAND1	Sheep	S292D	GST-CAND1 (5-245 of human CAND1)	
Cdc53	Sheep	S251D	GST-Cdc53 (1-288 of S. cerevisiae)	
Rabbit anti-NEDD8	Rabbit	R2911	Human NEDD8 (11-32) CKEIEIDIEPTDKVERIKERVEE	x
Rub1.1	Sheep	S105D	Yeast Rub1 (21-35) CDLVYHIKELLEEKEG	x
Rub1.2	Sheep	S105D	Yeast Rub1 (44-58) CIFQGKQIDDKLTVTD	x
Sheep anti-NEDD8	Sheep	S203D	Human NEDD8 (11-32) CKEIEIDIEPTDKVERIKERVEE	x

Table 2.5. *Antibodies produced by DSTT for the Kurz laboratory. Antibodies produced for this project were validated by myself whereas the other antibodies were validated by other members of the laboratory.*

2.1.3 Yeast strains

The following single deletion yeast strains (Table 2.6) were obtained in BY4741 background from the haploid Open Biosystems/Thermo Yeast Mat a knock out collection (# YSC1053).

Miscellaneous	E3 Deletions	DUB deletions	
pep4	ubr1	ubp1	ubp11
E1 deletion	san1	ubp2	ubp12
uba3		ubp3	ubp13
E2 deletions		ubp4	ubp14
ubc2	ubc8	ubp5	ubp15
ubc4	ubc10	ubp6	ubp16
ubc5	ubc11	ubp7	yuh1
ubc6	ubc12	ubp8	otu1
ubc7		ubp9	otu2

Table 2.6. *Open Biosystems Kan KO strains used. List of the strains obtained from the Open Biosystems collection where single geneshave been replaced with a KAN resistance cassette. Obtained from the Mata KO collection for use in this project.*

Double deletion mutants were generated by PCR-based knockout methods. Strain *uba1-204* and the isogenic wild type (WT) were from Professor Raymond Deshaies (Division of Biology, California Institute of Technology, Pasadena, CA, U.S.A.). Strain *cdc34-2* and the isogenic WT were obtained from Thimo Kurz (University of Dundee). The double and quadruple E2 mutant strains, ubc4::Trp1 ubc5::Leu2, ubc6::His3 ubc7::Leu2, and ubc4::Trp1 ubc5::Leu2 ubc6::His3 ubc7::Leu2 and the isogenic WT were obtained from Professor Mark Hochstrasser (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, USA).

Background	Genotype	Source
S288C	ubc12::Nat rub1::Kan	Thimo Kurz
BY4741	ubp3::Kan rub1::Nat	This project
BY4741	ubp3::Kan uba3::His	This project
S288C	pdr5::Kan rub1::Nat	This project
S288C	uba3::His	Thimo Kurz
S288C	rub1::Nat	Thimo Kurz
S288C	Wild Type	Thimo Kurz
W303	Wild Type	Thimo Kurz
MHY501	ubc4::Trp1 ubc5::Leu2	Mark Hochstrasser
MHY501	ubc6::His3 Ubc7::Leu2	Mark Hochstrasser
MHY501	ubc4::Trp1 ubc5::Leu2 ubc6::His3 ubc7::Leu2	Mark Hochstrasser
MHY501	Wild Type	Mark Hochstrasser
W303	uba1-204	Ray Deshais
W303	Wild Type	Ray Deshais
W303	cdc34-2	Thimo Kurz

Table 2.7. *Yeast strains from other sources. These strains do not come directly from the Open Biosystems yeast KO library. Most were generated using PCR mediated substitution techniques. The two temperature sensitive (TS) strains uba1-204 and cdc34-2 were grown at 25°C and shifted to the restrictive temperature of 37°C as required by experiments.*

2.1.4 Buffers

Buffer	Composition
1,10-Phenanthroline (OPT) (Aldrich)	200 mM stock was made from powdered 1,10-Phenanthroline and ethanol and kept at -20° for up to 1 month and used as needed
20% (w/v) glucose	20% (w/v) glucose in water; filter sterilized
3X reducing sample buffer	62.5M Tris pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 6M urea, 5% (v/v) beta-mercaptoethanol, bromophenol blue added to colour.
50% glycerol	50% glycerol, 50% water (autoclaved to ensure sterility)
DNA extraction buffer	2% (v/v) Triton, 1% (w/v) SDS, 100mM sodium chloride, 1x TE
Farmer's Reducer	30mM Potassium hexacyanoferrate (III), 30mM sodium thiosulphate (made fresh just before use)
Gel Extraction Buffer	5% formic acid, 50% acetonitrile
Homemade gel transfer buffer	48mM Tris base, 39mM glycine, 20% methanol
Lithium acetate mix	100mM lithium acetate in 1x TE
Lysis Buffer A (cultured cells)	50 mM Tris pH 7.4, 250 mM NaCl, 0.1% NP-40, 10% glycerol, 2 mM OPT, 10 mM IAA, Complete mini protease inhibitor from Roche
Lysis buffer B (cultured cells)	50 mM Tris pH 7.4, 250 mM NaCl, 0.1% NP-40, 10% glycerol, 10 mM IAA, Complete mini EDTA free protease inhibitor from Roche
Lysis buffer C (cultured cells)	50 mM Tris pH 7.4, 250 mM NaCl, 0.1% NP-40, 10% glycerol, 10 mM IAA, Complete protease inhibitor from Roche in 5x excess
MilliQ water (MilliQ)	water filtered through a Milli-Q Biocel system (Millipore) with a Quantum EX Ultrapure Organex cartridge (Millipore)

Nondenaturing lysis buffer for yeast	2 mM OPT, 10 mM IAA, 50 mM Tris pH 7.4, 250 mM NaCl, 10% (v/v) glycerol, 0.1% NP-40, complete protease inhibitor (5x excess)
Nonreducing sample buffer	320 mM Tris pH 6.8, 8% (w/v) SDS,32% (v/v) glycerol, 0.01% Bromophenol blue
One step yeast transformation buffer	250mM lithium acetate, 47% (w/v) polyethylene glycol 3550
PBST blocking buffer	12 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH7.4, 0.1% (v/v) Tween-20
PCR master mix (per tube using high fidelity polymerase kit from Roche)	0.5 µL genomic DNA, 35.25 µL MilliQ water, 0.5 µL 400 mM dNTP, 5 µL high fidelity buffer + $MgSO_4$, 5 µL 50% DMSO, 0.75 µL TAQ high fidelity DNA polymerase, 1.5 µL mM forward primer, 1.5 µL mM reverse primer
PEG mix	40% (w/v) Polyethylene glycol 4000 dissolved in lithium acetate mix
PPAD lysis buffer	50mM Tris pH 7.5, 150mM sodium chloride, 1% (v/v) Triton, 1mM EDTA, 1mM EGTA, 2mM TCEP, 1µM PefaBloc, 0.01% (w/v) leupeptin
PPAD PreScission protease buffer	50mM HEPES pH 7.0, 150mM sodium chloride, 1 mM DTT, 10% (v/v) glycerol
RIPA Buffer (for tissues)	50 mM Tris pH 7.5, 1 mM EDTA, 1% (v/v) Triton X-100, 150 mM NaCl, 10 mM IAA, 2 mM OPT, complete mini EDTA free protease inhibitor
TAE	40mM Tris acetate, 1mM EDTA
TBST blocking buffer	50mM Tris, 150mM sodium chloride, 0.1% (v/v) Tween-20, 5% (w/v) dried skimmed milk, 1% (w/v) BSA
TE buffer	0.1M Tris pH 7.5, 10mM EDTA
Transfer buffer (commercial gels)	1x Transfer buffer (invitrogen), 20% ethanol
Tris/HCl buffer solutions pH range 6.8-8.8	Tris buffers were adjusted to the required pH using concentrated (37%) HCl solution.
Tris-base buffer	0.1M Tris-base pH 9.2, 10mM DTT
Ubiquigent Buffer (PPAD Storage buffer)	50 mM HEPES pH 7.5, 150 mM sodium chloride, 1 mM DTT, 10% (v/v) glycerol

Wash buffer (TBST)	50mM Tris, 150mM sodium chloride, 0.1% (v/v) Tween-20
Yeast IP wash buffer A	125 mM NaCl, 1% NP-40, 120 mM Tris HCl pH 7.4
Yeast IP wash buffer B	250 mM NaCl, 1% NP-40, 120 mM Tris HCl pH 7.4
Yeast IP wash buffer C	500 mM NaCl, 1% NP-40, 120 mM Tris HCl pH 7.4

Table 2.8. *General Buffer recipes.*

Component	Function
EDTA	Chelates divalent cations
glycerol	protein stabilization
Iodoacetamide	alkylating agent to block disulfide bond formation
NaCl	Maintain ionic strength of buffer
NP40	nonionic detergent less harsh than SDS but can still help with membrane disruption and protein solubilization
OPT	metalloprotease inhibitor
Phosphatase inhibitor tablet	Inhibits phosphatase activity
protease inhibitor cocktail	inhibit aspartic-, metallo-, serine-, and cysteine-proteases
Tris pH 7.5	buffer to maintains near physiological pH
Triton	protein solubilization

Table 2.9. *Lysis buffer component functions.*

2.1.5 Mouse Tissues

Mouse tissues were obtained from several sources. Tissues panels were provided by Esther Sammler and Susanne Bandau both of the University of Dundee. Testes were obtained from Esther Sammler, Francisco Inesta-Vaquera, Vanessa Houde, and Ana Belen Perez-Oliva of Dr. Dario Alessi's Laboratory, University of Dundee. Additional brains and livers were collected and donated by Brian Dill. All organs were frozen in liquid nitrogen immediately after harvesting and stored at -80° until lysis.

2.2 Methods

2.2.1 Common laboratory techniques

2.2.1.1 Estimation of protein concentration by the Bradford method

The Bradford assay first described by (Bradford, 1976)makes use of the shift of Coomassie Brilliant Blue G-250 dye from 465 nm (red spectrum) to 575 nm (blue spectrum) upon binding to basic amino acids such as arginines, lysines, and histidines. For this project, protein concentration was estimated using 500 µLCoomassie protein assay reagent (Thermo) diluted with 500 µL of water. Lysis buffer alone was added as the blank whereas a serial dilution of purified BSA was used to generate a standard curve. Absorbance was measured at a wavelength of 595 nm a using an Ultrospec2100 pro (Amersham biosciences). Proteins were diluted to fall within the linear range. Bradford measurements were done in triplicate.

2.2.1.2 Estimation of protein concentration by the bicinchoninic acid assay

The bicinchoninic acid (BCA) assay described originally by Smith, 1985, was done using a kit adapted by Thermo and measurements were made using an EnVision 2104 Multilabel reader (Perkin Elmer).

2.2.1.3 Estimation of DNA concentration

DNA concentrations were analyzed by applying 2µL to a nanodrop (NanoVue version 4282.V2.0.3, General Electric). Pure samples were defined by an A260/280 ratio higher than 1.8.

2.2.1.4 Agarose gel electrophoresis of DNA

To separate DNA by molecular weight (MW) DNA gels were run on a mini-subcell GT system (BioRad). To pour these gels, 1% (w/v) agarose in 1X TAE was heated to boiling in a microwave, then allowed to cool slightly with constant stirring. Agarose

was poured into the mold and 2 µL ethidium bromide was added. Samples were suspended in 6X DNA loading dye (Promega). Samples were then loaded alongside a 1 kB Quick-Load DNA Ladder (New England Biolabs) and electrophoresed using the mini-subcell GT system (BioRad) at 90V for 40-60 minutes and visualized using a transilluminator (Molecular Imager Gel Doc XR+ Imaging System, Biorad).

2.2.1.5 Polymerase Chain Reaction (PCR)

For amplification of a gene typically 5 x 50 µL PCR tubes were prepared from a master mix. For confirmation of gene deletion, typically 1 tube was used. A typical mix made with the high fidelity polymerase kit from Roche is listed below (Table 2.10)

Typical PCR mixture	µL
Hi-FI buffer +MgSO4	5
400 mM dNTPs	0.5
50% DMSO	5
TAQ Hi-FI DNA polymerase	0.75
3.3 mM Forward primer	1.5
3.3 mM Reverse primer	1.5
MilliQ Water	35.25
genomic DNA	0.5

Table2.10*. Typical PCR mixture for one tube.*

A BioRad DNA Engine Peltier Thermal Cycler was used for PCR reactions. Conditions normally used were as follows:

Step 1: 95° for 5 minutes

Step 2: 95° for 1 minute

Step 3: 55° for 45 seconds (anneal)

Step 4: 68° for 1 minute/kB (amplify)

Step 5: Repeat from step 2 for 25-35 cycles

Step 6: 72° for 10 minutes

Step 7: 9° Final hold

The success of the PCR reaction was analyzed using a 1% agarose gel. If the target gene presence or deletion was confirmed that strain would be used for further experiments. For gene amplification experiments the 5 tubes were then pooled and purified using the Qiagen PCR cleanup kit.

2.2.1.6 Protein visualization

2.2.1.6.1 Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were diluted in 3x reducing sample buffer or 4x nonreducing sample buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the XCell SureLock Mini-Cell Electrophoresis System with Biorad PowerPac HC. Typically, NuPAGE 4-12% Bis-Tris Gels with 10, 12, or 15 wells (Invitrogen) were run in MES (Invitrogen) or MOPS (Invitrogen) buffers. For 3HA - Rub1ΔGG overexpression chase experiments in the *pep4Δ* and single E2Δ strains, and for the characterization of the Rub1 antibody, samples were run on 10-20% tricine gels with 1X Novex tricine SDS running buffer (Invitrogen).

Homemade gels were made using the Fisherbrand Gel tank complete supercooled for precast or handcast gels (formerly from SciPlus). These gels were poured with the following composition (Table 2.11). For gradient gels, the lower and higher percentage separating gel solutions were mixed using a gradient former. Gels were run in TGS buffer at 36mA, 300V.

Separating Gel	4.5% Stacking Gel	TGS
375mM Tris pH 8.8	4.5% acrylamide	25mM Tris pH 8.6
0.1% SDS	125 mM Tris pH 6.8	192mM glycine
0.1%APS	0.1% SDS	0.1% SDS
0.001% TEMED	0.1%APS	
	0.001% TEMED	

Table 2.11. *Recipes for homemade polyacrylamide gel componentsand running buffer. The separating gel contained the appropriate amount of acrylamide (typically 10% although 12%, 6-18% and 4-12% gels were also run).*

2.2.1.6.2 Gel Staining

Two methods for directly staining gels from SDS-PAGE were used; (1) Silver staining (Silver Stain for Mass Spectrometry, Pierce) or (2) Coomassie staining. Silver staining was accomplished as directed by the manufacturer. There were two methods of Coomassie staining utilized. First, instant stain was used as directed by the manufacturer (Instant Blue, Expedeon) for gels produced during optimization that were not to be sent for MS analysis. However on important gels bound for MS analysis, a homemade stain procedure was used because of its higher sensitivity. In this method, gels were fixed in 40% methanol 10% acetic acid for 20 minutes then rinsed with water and incubated in a solution containing 0.08% Coomassie Brilliant Blue G250, 1.6% ortho-phosphoric acid, 8% ammonium sulphate, and 20% methanol overnight. Gels were destained with water or 1% acetic acid. Gels were scanned using an Epson

Perfection V700 PHOTO scanner (Hertfordshire, UK). Contrast and brightness were in some cases altered using Photoshop (Adobe, San Jose, California) while making figures.

2.2.1.6.3 Transfer of proteins to nitrocellulose membranes

Following SDS-PAGE of commercial gels, proteins could be transferred using an X-Cell Sure Lock Mini-Cell Blot system (Invitrogen) packed as follows:

Cathode

3 Sponge pads

Filter paper

Filter paper

Protein gel

Nitrocellulose membrane

Filter paper

Filter paper

3 sponge pads

Anode

For commercially poured gels, the X-Cell Sure Lock system was then filled with NuPage transfer buffer (Invitrogen) at 30-35V for 1 hour to 1 hour and 15 minutes on a Biorad PowerPac HC. The only difference for homemade gels was the composition of the transfer buffer and, as the gels were thicker, transfer time was increased to 1.5 hours.

Protein transfer was confirmed by rinsing the membrane with MilliQ filtered water then applying ATX Ponceau S red staining solution (Fluka Analytical). The membranes

were then rinsed with MilliQ water again until the background was nearly white. Areas where protein had transferred were then visible as pink bands. Areas without protein remained white. Membranes were placed within a page protector and scanned on an Epson Perfection V700 PHOTO scanner.

2.2.1.6.4 Immunoblotting/Western Blotting

To perform a Western Blot (WB), proteins are first transferred from polyacrylamide gels to nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5% milk 1% BSA in TBST for at least one hour then incubated with primary antibody in 5% milk 1% BSA in TBST for one hour to overnight. Primary antibodies and secondary anti protein G were typically saved after use at -20°C and thawed for reuse. After primary incubation was complete, the membrane was washed 3-4 times with TBST for 5-10 minutes. Secondary antibody was then added at 1:5000-1:10000 for ~ 45 minutes. The membrane was then rinsed twice with TBST and washed n TBST 3 times 10 minutes and once for 5 minutes. The HRP conjugated antibodies were incubated with Millipore Immobilon Western chemiluminescent HRP substrate for enhanced chemiluminescence (ECL) for 1.5 minutes. Amersham hyperfilm ECL from GE was then exposed to the membranes in a dark room and the films were developed using an SRX-101A Medical Film Processor (Konica Minolta, Tokyo Japan). Films were scanned using the Epson scanner. Contrast and brightness were in some cases altered using Photoshop (Adobe, San Jose, California) while making figures.

2.2.1.6.4.1 Exceptions to typical WB procedure

In order to develop a blot where the primary antibody was already conjugated to HRP (as with HA-HRP), membranes are blocked as above then incubated with the primary

antibody overnight. Membranes are then washed 4 times for 10 minutes in TBST and developed as described above.

The sheep anti-Rub1 antibody has many nonspecific interactions and worked most efficiently if the heavily banded area (20-60 kD) of the membrane was removed. The bottom portion of the membrane (free Rub1) and the high molecular weight region (the cullins) were then Western Blotted using PBST based buffers. Anti-Rub1 antibody was used 1:500 overnight and could not be reused.

In order to increase the signal for lower molecular weights forms of ubiquitin and ubiquitin conjugates (monomer dimer etc), the nitrocellulose membrane was boiled in water for 30 minutes prior to blocking and the primary Dako anti-ubiquitin antibody was incubated for 1-2 hours at RT. The rest of the procedure is as above.

2.2.1.6.4.2 Membrane stripping

If the membrane used for a WB had bands in an area that needed to be reprobed with another antibody (for example to obtain the image for the loading control), the Abcam procedure for Medium stripping was used (Abcam). The only difference to the procedure described is that during the second wash with stripping buffer, the membrane was warmed slightly in the microwave before being allowed to incubate for 5-10 minutes.

2.2.1.6.5 Dot blot

To test antibody reactivity to purified protein, serial dilutions of the target protein were made both native (in Ubiquigent buffer) and denatured (In 3x reducing sample buffer).

To control for nonspecific binding at least one other protein was treated in the same manner. All proteins to be analyzed were then applied to a nitrocellulose membrane (GE Healthcare) in 1-5 μL aliquots. The membrane was subsequently blotted as with a normal WB using primary antibody dilutions of 1:500 overnight.

2.2.1.7 Antibody production schemes

The antibodies generated for this project were tested for specificity and possible uses as follows.

2.2.1.7.1 Rub1 polyclonal antibody

Peptides to be used for this project were selected by Thimo Kurz. A single sheep was immunized against yeast Rub1 residues 21-35 and 44-58. Three bleeds of Sheep-anti-Rub1 were purified against the antigens. Antibodies were then tested by dot blot to determine if they specifically recognized Rub1 without ubiquitin crossreactivity. As the antibody purified against Rub1 residues 44-58 displayed some crossreactivity with ubiquitin, the other Rub1 antibody (purified against residues 21-35) was used. Antibodies were tested for efficacy via WB of whole cell lysate. The third bleed was also tested for immunoprecipitation (IP) capability.

2.2.1.7.2 NEDD8 polyclonal

A sheep and a rabbit were immunized against residues 11-32 of human NEDD8. Serum purified against the antigen was checked by dot blot for NEDD8 specificity, cross reactivity with ubiquitin, and crossreaction with high levels of protein using BSA. Subsequently these antibodies were also tested against WB of HCT-116 lysate. WB specificity was confirmed by comparing samples with and without inhibition of the

NAE using MLN4924 treatment. If the intensity of WB bands decrease following inhibition of the neddylation machinery they are likely genuine substrates. In these initial dot blots and WBs of the NEDD8 polyclonal, results were compared to signal obtained using Mil10, a highly specific rabbit monoclonal anti-NEDD8 from Millenium Pharmaceuticals. Attempts to use the polyclonal antibodies for IP were made using the DSS crosslinker kit (Pierce), the AminoLink Plus Immobilization Kit (Pierce), NHS-activated M-PV magnetic beads (Perkin Elmer) and crosslinking free experiments.

2.2.1.8 Common bacterial protocols

2.2.1.8.1 Transformation of *E. coli*

DH5α cells were prepared by the DNA Cloning Service (University of Dundee, Scotland) as described by Inoue et al., 1990. Competent cells were stored at -80°C and thawed as needed on ice. In a microcentrifuge tube, 1µL of plasmid was combined with100µL of bacteria and allowed to incubate on ice for 30 minutes and then heat shocked for one minute at 42°C. The bacteria were returned to ice for 2 minutes then plated on an LB-AMP and allowed to grow at 37°C.

2.2.1.8.2 Isolation of DNA from bacteria

3mL of LB-Amp was inoculated with *E. coli* carrying the target plasmid and allowed to grow overnight shaking at 37°C 200 RPM. It was subsequently purified using the QIAprep Spin Miniprep Kit (Qiagen) using the provided elution buffer and concentration was measured using the nanodrop method. Plasmids were then stored at -20°C and thawed as needed.

2.2.1.8.3 Preparation of bacterial glycerol stocks

Glycerol stocks were made by combining 1mL of *E. coli* grown in LB-Amp liquid media overnight at 37°C with 1mL of 50% glycerol which had been autoclaved. Starter cultures were prepared from this stock by scraping an inoculating loop across the surface of the stock and restreaking the bacteria onto fresh LB-Amp plates.

2.2.1.9 Common yeast protocols

2.2.1.9.1 Growth of yeast

Yeast was grown on rich YPAD media (yeast extract-peptone-dextrose-plus Adenine) or in synthetic dextrose media (SD). Selection for knock out (KO) strains and strains carrying expression plasmids were maintained on plates with the appropriate selection marker. All wild type strains were grown on YPAD. Yeast grown on agar could be saved at 4°C with parafilm around the plate for up to 1 month before being restreaked on a fresh plate.

Yeast grown in liquid media was started as a preculture by inoculation of 5-10 mL of the appropriate media (KO strains and strains carrying expression plasmids in media with the appropriate selection marker and, wild type in YPAD) with one colony from the plated yeast and grown overnight at standard shaking incubator conditions (Infors HT Multitron at 200RPM, 30 °C for all strains except temperature sensitive (TS) strains which were grown at 25°C). The following day, the OD_{600} was measured and the yeast was diluted to the desired OD and volume before being returned to standard shaking incubator conditions. TS strains were shifted to the restrictive (37°C) temperature for 1hour to induce TS mutation.

2.2.1.9.2 Measuring yeast growth in liquid media

Yeast concentrations correlate to the optical density (OD) of cultures at a 60 nm wavelength. Yeast cultured overnight obtains very high densities which are outside the accurate range of the OD_{600} /concentration correlation. In order to more accurately record cell density, overnight cultures were diluted 1:10 in the medium they were cultivated in. Cells harvested from the exponential growth phase did not require dilution. Blanks were prepared using uninoculated medium. OD_{600} was measured using an Ultraspec 2100 pro (Amersham Biosciences).

2.2.1.9.3Trichloro acetic acid (TCA) protein preparation from yeast

6 mL of yeast was pelleted and incubated on ice or at -20°C until all samples for a specific experiment had been collected. The pelleted yeast was lysed in ice-cold 1.85 M sodium hydroxide and 7.6% beta-mercaptoethanol and incubated on ice for 10 minutes. An equal volume of 50% TCA solution was then added. Then samples were incubated for 10 minutes on ice to allow for proteins to precipitate. Samples were pelleted at 4°C, washed in ice-cold acetone and pelleted again. 10µLof 1M Tris pH 7.5 was then added to the samples. Finally, samples were resuspended in 3x reducing SDS sample buffer or Novex Tricine SDS Loading buffer and boiled for 5 minutes at 95°C. These samples could be loaded directly on a gel. Alternatively these samples could be stored at -20°C until such a time as they could be thawed/boiled (5 minutes 95°C) and loaded on a gel.

2.2.1.9.4Freezer Mill preparation of proteins from yeast

Yeast was grown in liquid culture then pelleted and resuspended in nondenaturing lysis buffer for yeast (2 mM OPT, 10 mM IAA, 50 mM Tris pH 7.4, 250 mM NaCl, 10% (v/v) glycerol, 0.1% NP40, complete protease inhibitor (5x excess)). The resuspended

yeast was flash frozen in a drop-wise manner in liquid nitrogen. In this form yeast was either lysed immediately or stored at -80°C for up to 4 days before lysis. Upon removal from -80°C storage, yeast was returned immediately to liquid nitrogen then lysed using the Freezer Mill 6770 Spex Sample Prep precooled with liquid nitrogen with the following settings: 4 cycles with a 1 minute precool, 2 minute run, 1 minute cool time, and 5CPS rate. The yeast was then thawed on ice and diluted with fresh lysis buffer. Protein concentration was measured using the Bradford Assay and samples were adjusted with lysis buffer such that all samples within one experiment were of equivalent concentration.

2.2.1.9.5Genomic DNA preparation

In order to prepare genomic DNA from yeast, 5mL of the appropriate selective media was inoculated with the target strain and grown overnight in a shaking incubator under standard conditions. The stationary phase culture was then centrifuged at 2791 x g for 5 minutes and the supernatant was discarded. The pellet was resuspended in 1mL of TE1X, transferred into a microcetnrifuge tube, and pelleted at 17000xg for 1min in a RT benchtop centrifuge. The yeast pellet was combined with 200µL of DNA extraction buffer, approximately 0.3g of acid-washed glass beads (Sigma), and 200µL of biophenol (BDH) and vortexed for 4 minutes to break up the DNA. Then 200µL of TE 1X was added and the tubes were centrifuged at 17000xg for 5minutes at room temperature (RT) in the desktop centrifuge. DNA was precipitated from the aqueous phase with 1mL of ice-cold 100% ethanol. The tubes were then incubated at -20°C for 30 minutes. The solution was centrifuged at 17000xg for 2 minutes at RT and the supernatant was discarded. The DNA pellet was washed with 1mL of ice-cold 70%

ethanol and centrifuged again at 17000xg for 2 minutes at RT. The ethanol supernatant was again discarded. The pellet was allowed to dry under the hood for 10-15 minutes at RT before being resuspended in 50µL of MilliQ water. The DNA/water solution was allowed to stand at RT for approximately 10 minutes before concentration was measured by nanodrop and DNA was frozen at -20°C until needed.

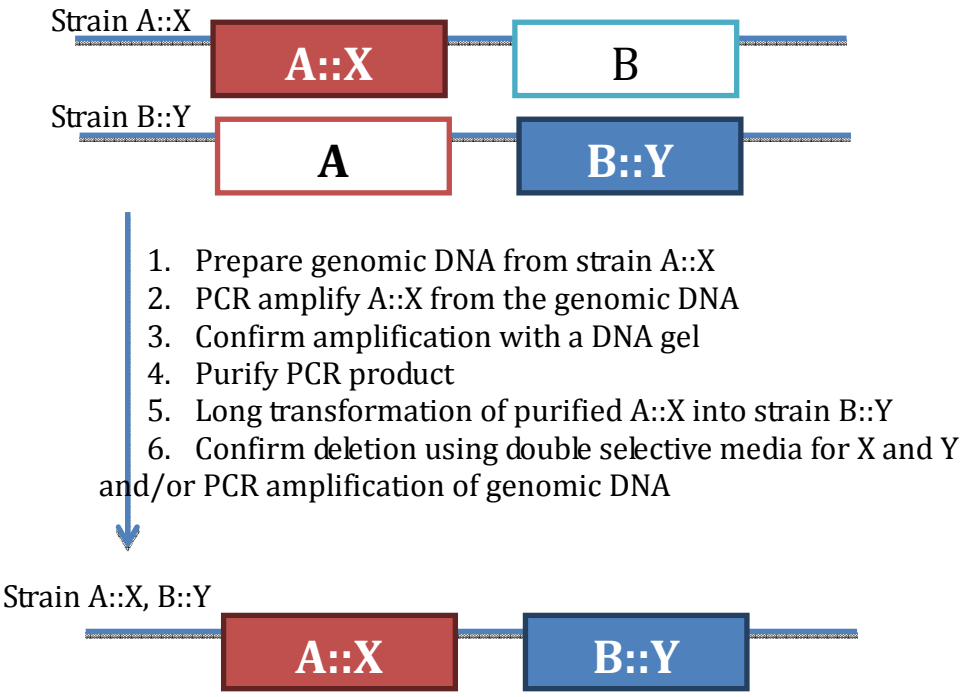


Figure 2.1. Homologous recombination of PCR products into yeast.

2.2.1.9.6Long yeast transformation protocol for homologous recombination

Conveniently, the single deletions of all genes we sought to make double deletions of for this project were already within the Kurz laboratory library. Two deletion strains could therefore be grown and a PCR based method could be used to amplify and transform one deletion into a background strain bearing the other deletion and thereby generate the double deletion strain (Figure 2.1).

In order to accomplish this, the genomic DNA of the mutant strain was first prepared. The resistance cassette which replaced the gene of interest was amplified via PCR along with ~250bp of the 3'UTR and 5'UTRs immediately adjacent to the gene. The PCR product was then pooled and purified. The strain where the gene was to be deleted was grown in 5mL of the appropriate yeast media overnight shaking at 30°C. The culture was then diluted 2mL into 50mL and allowed to grow to exponential face (OD_{600} 0.4-0.8). The yeast was harvested via centrifugation at 1570 x g for 5 minutes at RT. The supernatant was discarded and the pellet was resuspended in 500µL of Lithium acetate (LiAC) mix. Salmon Sperm DNA was boiled for 5 minutes prior to transformation. For each transformation, 100µL of the yeast/LiAC Mix is combined with 30µL of the purified PCR product, 10µL salmon sperm DNA and 700µL of PEG mix. The mixture was then allowed to rotate at RT for 30 minutes. The yeast was subsequently heat shocked for 15 minutes at 42°C and centrifuged at 9000 x g for 2 minutes at RT. The pellet was resuspended in 200 µL YPAD and allowed to incubate rotating at RT for 2-3 hours.

The yeast was then plated on the appropriate restrictive media and allowed to grow for several days at the appropriate temperature. Of the colonies that grew following transformation, 6-8 were restreaked on the appropriate double restrictive media agar to confirm that the yeast really carried the double deletion. For important strains, this confirmation was augmented with PCR amplification of the deleted gene to prove that homologous recombination was successful.

2.2.1.9.7Transformation of plasmids into yeast

Plasmids can be simply inserted into yeast by combining a mixture of 85 µL of One Step Buffer (250mM lithium acetate, 47% (w/v) polyethylene glycol 3550) with 100 mM DTT, 50 ng of plasmid DNA and 50 µg of freshly boiled salmon sperm DNA. The mixture was inoculated with a colony of the appropriate parental strain. The mixture was then vortexed and incubated for 30 minutes at 45°C. The yeast was plated immediately following heat shock on the appropriate restrictive media. Colonies were allowed to grow at 30°C. Resulting colonies are restreaked on plates with the appropriate selective media. For TS strains, the only difference is that once plated, the yeast is allowed to grow at 25°C and 6 colonies are subsequently restreaked onto two plates. One plate is allowed to grow at the permissive temperature (generally 25°C). The other is grown at the restrictive temperature 37°C. The strain grown at the permissive temperature should grow while the yeast grown at the restrictive temperature should die. If this phenotype is not observed, then there has been an secondary mutation that eliminates the temperature sensitivity of the strain.

2.2.1.9.8 Preparation of yeast glycerol stocks

Approximately 3mL of YPAD was inoculated with the yeast to be frozen and allowed to grow overnight typically at 30°C (TS strains 25°C). Of that culture, 1mL was combined with 1mL of 50% sterile glycerol in a cryovial and frozen at -80°C. Strains were restreaked from these stocks onto plates of the appropriate restrictive media.

2.2.1.9.9 Collection of yeast samples

To obtain samples of different strains growing in the exponential phase, 5-10 mL of the appropriate restrictive media or YPAD was inoculated with yeast and allowed to grow

overnight (generally 30°C, TS strains 25°C). Strains were then diluted to an OD_{600} between 0.2-0.4 and allowed to at least double before 3-6 mL was collected with an OD_{600} between 0.4-0.8. Samples were pelleted and the supernatant was discarded. The yeast pellet was either TCA precipitated directly or frozen at -20°C for 1-3 days before TCA precipitation.

In experiments where collection was required at various time points, we similarly inoculated a preculture and grew it overnight. The yeast was then diluted to an OD_{600} of 0.2 and allowed to grow to an OD_{600} of 0.4. Strains were then treated (ex. galactose induction, cycloheximide treatment, or addition of MG132).

To grow yeast in liquid culture, 5-10mL of the appropriate liquid media was inoculated with yeast from the agar plate. This preculture was grown overnight at the appropriate temperature (generally 30°C, TS strains 25°C). The following day, precultures were diluted to an OD of 0.2-0.3 in the desired volume of media. For overexpression chases this volume was typically 50 mL while for strains that were being harvested purely for WB analysis of untreated exponentially growing cells, this volume was typically 10-20 mL. For the IP experiments this volume could be between 0.5L and 4L. Cultures were returned to the shaking incubator under standard conditions and allowed to at least double. Cultures were collected in exponential phase with an OD_{600} between 0.4-0.8.

Samples of 3-6 mL were pelleted and TCA precipitated or frozen at -20°C and TCA precipitated the following day.

Samples ≥0.5 L were spun down at 4°C, 4200 RPM for 2 hours and the OD_{600} of the supernatant was measured to confirm that the majority of yeast was in the pellet (OD of 0.1 or less for the supernatant). The supernatant was then discarded. The pellet was resuspended in 5-8 mL of water and transferred to a 50 mL centrifuge tube. The yeast was then repelleted in an Eppendorf centrifuge at 4000 RPM and resuspended in yeast lysis buffer. Samples were flash frozen in liquid Nitrogen and either lysed directly by freezer mill or stored at -80°C overnight and lysed the following day by freezer milling.

2.2.1.9.10 Proteasome inhibition in yeast with MG132

In order for the proteasome inhibitor MG132 to pass through the cell walls of the strains of interest, the drug pump *pdr5* gene was deleted. A 5-10mL preculture was inoculated and grown at standard conditions in the shaking incubator. Yeast was then diluted to an OD_{600} of 0.2 and allowed to grow under standard shaking incubator conditions. At OD_{600} of ~0.5, cultures were split in half. One half was treated with 50 μM MG132 and the other with an equivalent volume of DMSO. Cultures were allowed to grow for an additional hour at standard shaking incubator settings before being harvested, TCA precipitated, resuspended in 3x reducing sample buffer and run on SDS-PAGE.

2.2.1.9.11 Galactose induced overexpression chase assay

Yeast carrying pYES2 galactose inducible plasmids were grown in the appropriate restrictive raffinose based media (S_{Raff-} - URA). As raffinose is the most preferred sugar available in this media, the Gal promoter is not activated and the protein coded for by the plasmid is not expressed. Further the the Ura drop out allows for selection of yeastcarrying the plasmid. When the culture reaches an OD_{600} of ~0.4, expression of the protein is induced through addition of 2% final (v/v) galactose for 1 hour. An aliquot is

then saved as time point zero and expression is shut off by addition of 2% glucose. As glucose is a more preferred sugar, the Gal promoter is shut off and protein expression from the plasmid is ceased. At various time points thereafter samples were collected. All samples from a single time course were TCA precipitated and resuspended in sample buffer concurrently.

To chase protein levels in these samples, they were run on SDS-PAGE, transferred to nitrocellulose membranes and then Western blotted against the protein or the tag on the protein as indicated.

2.2.1.9.12 Cycloheximide chase assay

Cycloheximide is a drug that can inhibit ribosomes. It is used to stop translation and determine stability of various proteins. In order to use this drug on enogenous yeast proteins, strains were grown in rich media until reaching an OD_{600} of ~ 0.6. Then the culture was split in half. One half was treated with cycloheximide at a final concentration of 35 μg/mL and one half was treated with an equal volume of the vehicle control. Samples are collected at various time points after drug treatment and compared for Rub1 levels. For overexpression chase assays tested with cycloheximide treatment, the continued accumulation of the construct in WB of the vehicle control compared to the stability of Rub1 following cycloheximide treatment was used as a control for drug efficacy. However in treatment of non-overexpressed protein, the growth curve was used as a positive control. The sample treated with the ribosomal inhibitor does not continue to double while the sample treated with vehicle control continues to grow.

2.2.1.9.13 Near endogenous Rub1 level comparisons

Yeast was grown to exponential phase in the appropriate media under standard shaking incubator conditions. Then an aliquot was harvested, TCA precipitated, resuspended in sample buffer and then run on 4-12% Bis-Tris gels, transferred to nitrocellulose membranes, and Western blotted against Rub1 or HA as indicated.

2.2.1.10 Common cell culture protocols

Both U2OS and HCT116 cells were grown in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 2mM L-Glutamine. Cells were grown in T-75 flasks at 37°C under 5% CO_2 atmosphere and split when they reached 80-100% confluency.

2.2.1.10.1 Splitting cells

Medium was aspirated and cells were washed in prewarmed DPBS to remove residual medium. Cells were trypsinized using 3 mL of 0.05% Trypsin-EDTA for approximately 2 minutes. Cells were then diluted into fresh, prewarmed medium and seeded in new flasks to the desired density (typically cells were diluted 1:4).

2.2.1.10.2 Small interfering RNA (siRNA) transfection of cultured cells

In order to knockdown gene expression in cultured human cancer cells, siRNA was used. These oligomers interfere with the expression of genes bearing complementary nucleotide sequences. Therefore treating cells with UCHL3 siRNA should result in the specific silencing of that gene.

HCT116 and U2OS cells were grown in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 2mM L-Glutamine and plated at a density of approximately 1.2 million cells/100 mm dish and allowed to grow overnight. Typically, gene expression was knocked down using 20 nM siRNA so that will be used as the standard protocol example. In order to have a final volume of 4 mL at 20 nM siRNA, siRNA is diluted to 400nM in optimem. 195 µL optimem is mixed with 5 µL lipofectamine RNAiMAX. Equal volumes of these two mixtures are then mixed and well and allowed to incubate for 20-30 minutes. Then the siRNA/lipofectamine/optimem mixture is combined with media (400 µL/3600 µL) and added to the plate. The plate is then returned to the incubator and allowed to grow for the prescribed time (usually 72 hours) before harvesting for downstream testing (WB, FACS, or IP).

2.2.1.10.3 Flourescence activated cell sorting (FACS)

Cells destined for FACS analysis were grown in 10 cm dishes and treated with siRNA as indicated. Following the knockdown of target genes, samples were collected by trypsinization. Samples were then pelleted by centrifugation at 1000 RPM 4°C for 10 minutes. Media was removed and the pellet was washed with ice cold PBS. Cells were again pelleted. The pellet was vortexed as 1 mL of 70% ethanol was added drop-wise to the pellet. The samples were allowed to stand at -20°C for approximately 1 week. Cells were again pelleted using the same settings and washed twice with ice cold PBS. The cells were then resuspended in 300-500µL of staining buffer (50µg/mL propidium iodide, 50µg/mL ribonuclease A, in 1%BSA in PBS). The samples were transferred to FACS tubes covered in aluminium foil and protected from light at room temperature for

20-30 minutes. With the assistance of Dr. Yann Thomas, cells were analysed by flow cytometry using a FACS Calibur (Becton Dickinson) controlled using a Mac computer with Cell Quest data acquisition software. Results were analysed using FlowJo software.

2.2.1.10.4 MLN4924 treatment of cultured cells

MLN4924 is a selective inhibitor of the NAE and therefore the neddylation machinery in general (Milhollen et al., 2010; Smith et al., 2012). Cells were treated with either 2 μM MLN4924 or the equivalent volume of DMSO control for 8 hours.

2.2.1.10.5 Lysis of cultured cells

Cultured cells were lysed in several ways. First, and most preferably if samples were to be immediately run on SDS-PAGE, cells were lysed by direct addition of hot 3x reducing sample buffer. Cells were then scraped off in this buffer and DNA was fragmented by repeatedly flowing through a syringe. Samples were boiled for 5 minutes. Cells to be used for other downstream experiments such as pulldowns or IPs, were typically lysed in one of two ways. First, cells could be harvested by trypsinization washed with PBS and then lysed by adding lysis buffer and incubating on ice for 5-10 minutes while DNA was fragmented using a syringe. Second, cells could be harvested by placing the plates on ice, aspirating the media, washing cells with ice cold PBS and then adding ice cold lysis buffer and scraping cells off of the plate. The lysis buffer based lysis techniques required that the samples then be clarified at 17000 x g, 4°C for 15 minutes to remove cell debris. Either the whole or part of the sample was then destined for SDS-PAGE and was diluted with 3x reducing sample buffer and boiled for 5 minutes. The concentration of the lysate was determined with the BCA assay.

2.2.1.10.6 Check for degradation

Although a tool exists for purification of polyubiquitylated proteins (TUBES(Hjerpe et al., 2009)) there is currently no such tool for neddylated proteins. It would be especially useful to develop such a tool given current confusion in the literature over genuine endogenously neddylated proteins. Towards this end, Dr. Roland Hjerpe, the inventor of the TUBEs system, developed the theory required for the NEDD8affinity matrix (NAM). In order to circumvent the significant similarity between ubiquitin and NEDD8 and specifically target only neddylated proteins, Dr. Hjerpe posited that it might be possible to use a catalytically inactive form of NEDP1 (NEDP1 C163A) to pull on neddylated proteins. This system makes use of the high specificity NEDP1 has for NEDD8 thereby excluding ubiquitin.

First to confirm that such a construct pulled on NEDD8 but not ubiquitin, GST-NEDP1 C163A was used to specifically pulldown NEDD8 from an in vitro mixture of purified NEDD8 and ubiquitin. Dr. Hjerpe also performed a pilot experiment to see if such a construct could pull down neddylated proteins from cell lysate. I took over the project at this point and worked with various tags and attempted various protocol alterations to enhance the effectiveness of the matrix. All resin was preequilibrated with the appropriate lysis buffer and incubated on ice prior to the addition of any protein.

In order to use a GST tagged NAM, HCT116 cells were grown to confluency then lysed in a Lysis bufferA containing the probe protein (either GST, GST-NEDP1 C163A, or His-HA-GST-NEDP1 C163A). These lysates were clarified in a desktop centrifuge for

30 minutes at 4°C and high speed. Protein concentrations were determined using the Bradford Assay. Proteins were diluted such that the same quantity of protein was loaded for each point. In initial experiments, proteins were precleared on control agarose for 1 hour but in later work this was increased to 3 x 20 minutes on control agarose in order to reduce nonspecific binding of neddylated and ubiquitylated proteins to the final resin. The type of resin selected for each experiment depended upon the type of NAM tag. For the GST tagged NAM, Glutathione agarose beads were used and GST alone was used as a control. For the HA tagged NAM, mouse monoclonal anti-HA agarose beads (Sigma) were used and vehicle (Ubiquigent buffer) was used as a control. Proteins in lysis buffer A containing 5µM of thepurified NAM protein was incubated on the appropriate beads for 1-3 hours at 4°C. Beads were then washed three times with NAM wash buffer (50mM Tris, 150mM NaCl, pH 8.0) or DPBS and then eluted by heating the beads in a volume of Laemmli buffer equivalent to the bed volume.

To ascertain the relative success of these techniques the samples were run on 4-12% Bis-Tris SDS Page gels and transferred to nitrocellulose membranes. They were subsequently blotted for NEDD8 and ubiquitin.

2.2.1.11 Mouse tissue protocols

2.2.1.11.1 Tissue panel comparisons

In order to compare the neddylation profile between different tissues, a selection of various tissues were tested. These panels include tissues like heart, brain, testes, liver, and kidney. Samples to be used for tissue panels were lysed by Esther Sammler and then subsequently resuspended in denaturing sample buffer (samples for other uses were prepared by me and not immediately placed in sample buffer). SDS-PAGE was

performed using 4-12% Bis Tris gels. Proteins were then transferred to nitrocellulose membranes and Western Blotted using either Mil10 or Epitomics anti-NEDD8 antibodies.

2.2.1.11.2 Tissue lysis

Tissue lysate was prepared in RIPA buffer using a benchtop tissue homogenizer (Kinematica polytron PT 1200 E). Homogenized tissue was kept on ice for 15-30 minutes and passed through a precooled syringe several times. Homogenates were then clarified at high speed (17000 x g) at 4°C for 15 minutes. Protein concentration was identified using the BCA assay and samples were aliquotted into precooled eppendorf tubes, snap frozen in liquid nitrogen, and stored at -80°C until needed. When tissues were removed from storage they were allowed to thaw on ice.

2.2.2 Immunoprecipitation

All resins were preequilibrated with the appropriate lysis buffer and cooled on ice before protein was added to them. Samples were prepared for immunoprecipitation in a nondenaturing buffer (ex. RIPA buffer), as described in the relevant sections above (yeast, Section 2.2.1.9.4 ; cultured cells,Section 2.2.1.10.5; tissues, Section 2.2.1.11.2). Samples were either used directly after preparation or were stored at -80°C until needed and then thawed on ice in the cold room.

2.2.2.1 Native immunoprecipitation of yeast HA-Rub1

Each strain carrying HA-Rub1 to be immunoprecipitated was grown to exponential phase in 2-4 L (depending on the experiment) of the appropriate restrictive media (SD-His). The yeast was then pelleted by ultracentrifugation and washed with water before

being resuspended in nondenaturing lysis buffer for yeast (2 mM OPT, 10 mM IAA, 50 mM Tris pH 7.4, 250 mM NaCl, 10% (v/v) glycerol, 0.1% NP40, complete protease inhibitor (5x excess)) and flash frozen in liquid nitrogen. Frozen yeast was lysed by freezer milling under standard conditions. The frozen yeast powder was then thawed on ice and clarified at high speed (1700 x g) at 4°C for 20 minutes. Protein concentration was determined with the Bradford method. Yeast proteins were then diluted to approximately 140 mg of protein in 50 mL of lysis buffer and a small fraction was saved as input at this point. The remainder was precleared on 600 µL of control agarose beads for 2 hours while protected from the light. The control agarose beads were pelleted and the protein was transferred to a new 50 mL tube and incubated with 300 µL of mouse monoclonal HA agarose beads (Sigma) for 10-16 hours. The flow through was collected by centrifugation. Beads were washed using a graded series of wash buffers of different salinity in the following order: Yeast IP wash buffer A, B, C, B, A. All washes were 10mL for 10 minutes while rotating.

Proteins were eluted by addition of 8 M urea (3 x 300 µL shaking at 37.5°C on an eppendorf thermomixer at 300 RPM). The beads were then resuspended in 8 M urea and split into two. The beads were pelleted again and the urea based supernatant was collected and added to the pool of the first three urea elutions. One half of the remaining beads was boiled in 3 x reducing sample buffer while the other half was boiled in 4x nonreducing sample buffer for 5 minutes. The urea elution pool was centrifuged again to remove any possible beads that might have been aspirated by accident. An Amicon Ultra “ultracel 5K” (Millipore) was pre-wet with200 µL of 8 M urea and then the urea elution pool was added to it. The spin filter was spun at 3100 x g

for 40 minutes in the eppendorf centrifuge at 4°C. There was about 70 µL final urea elution volume and 35 µL of reducing sample buffer was added to it. The urea elution pool was then boiled at 95°C for 5 minutes. Samples were frozen at -20°C overnight.

A small amount of the samples (5%) was run on a mini 4-12% BisTris gel (Invitrogen), transferred to a nitrocellulose membrane and blotted with the HA-HRP antibody. The remainder of the samples was run on a large homemade 10% gel to separate proteins between 25 and 250 kD. This gel was silver stained and bands from all three elution conditions were cut for mass spectrometric analysis.

2.2.2.2 Native immunoprecipitation of untagged proteins

For native/non-denaturing immunoprecipitation of non-crosslinked antibody against untagged protein, proteins were precleared 3 x 20 minutes on control agarose (Sigma) then loaded onto protein G agarose along with the desired quantity of antibody (this amount will be noted in all associated figure captions). This mixture was incubated in the cold room for 1 hour to overnight. The beads were washed using the following series of buffers: 3 x lysis buffer, 1 x water, 1 x lysis buffer. Protein was then eluted from the beads by addition of 3x reducing sample buffer and boiled at 95°C for 5 minutes. The beads were then pelleted and the supernatant could be used for SDS-PAGE.

2.2.2.3 Denaturing immunoprecipitation

All denaturing mammalian IPs were done following the procedure established by LeonandHaguenauer-Tsapisas adapted fromKragt et al., 2005,the only exceptions being that 200 mM OPT and 100 mM IAA were added to all 6 buffers and washes were done

for 3-5 minutes with gentle rotation. Elution from resin was accomplished by direct addition of a volume of 3x sample buffer followed by boiling at 95°C for 5 minutes.

2.2.3 Mass Spectrometry (MS)

All samples analyzed using mass spectrometry (MS) were collected by in gel digestion from silver or Coomassie stained polyacrylamide gels.

In-gel protein alkylation and digestion of silver stained gel slices

Bands were extracted from silver stained polyacrylamide gels using a stringently cleaned scalpel, cut into small fragments, and deposited in individual Eppendorf tubes. Gel slices were washed 3 times with milliQ water. Silver staining was reduced with Farmer's Reducer (30 mM Potassium hexacyanoferrate (III), 30 mM sodium thiosulphate (made up fresh)) for 5 minutes then washed with MilliQ water until gel pieces became clear (typically 3 times 5 minutes). Samples were equilibrated with 100 mM ammonium bicarbonate (ABC) (Fluka) for 20 minutes with gentle agitation, washed with 50mM ABC/50% acetonitrile (ACN), and rinsed with ACN (Millipore). The supernatant was removed and samples were allowed to air dry (for approximately 20 minutes). Samples were reduced with 10mM dithiothreitol (DTT) (Formedium) in 100mM ABC for 30 minutes at 56°C. DTT was discarded and samples were alkylated with 50mM iodoacetamide (IAA) (Sigma) in 100mM ABC for 30 minutes at room temperature in the dark. Gel slices were then washed twice with 50mM ABC/50% ACN for 15 minutes shaking at 500 RPM on an eppendorf thermomixer. Samples were then rinsed with 100% ACN and dehydrated with 500µL 100% ACN for 20 minutes with gentle agitation. Samples were digested in 0.02 µg/µL sequencing grade modified trypsin (Promega) in 50mM ABC for one hour on ice followed by 37°C overnight at

500RPM. The supernatant was saved. To maximize peptide collection from the gel slices, samples were then sonicated in gel extraction buffer (5% formic acid, 50% ACN) for 15 minutes. The gel extraction step was repeated 3 times. The supernatant pool was precooled on dry ice before being dried by a Savant SPD131 DDA SpeedVac Concentrator (Thermo). Samples were then cleaned on C18 columns (Nest Group).

In gel alkylation and digestion of Coomassie stained gel slices for mass spectrometry

Bands from polyacrylamide gels with Coomassie staining could be similarly picked with a scalpel and cut into fine pieces. These gel slices were washed with water, then 50% ACN, then 0.1 M ABC, then 50%ACN/50 mM ABC. The gel slice was then reduced using 10mM DTT/0.1 M ABC at 37C for 20 minutes and in gel alkylated using 50 mM IAA/0.1 M ABC in the dark for 20 minutes at RT. Gel pieces were then washed with 50 mM ABC followed by 50 mM ABC/50% ACN. If the gel bands were not colourless at this point, the last wash was repeated as long and as often as necessary (usually only 2 washes with the 50 mM ABC/50% ACN was needed). Once colourless, the gel pieces were dehydrated with ACN for 15 minutes. The supernatant was removed and the pieces were Speed-Vaccumed to dryness. The gel pieces were then swollen in 25 mM Triethylammonium bicarbonate (TEABC) containing 5 µg/mL of sequencing grade modified trypsin (Promega). Gel slices were then allowed to incubate at 30°C for 30 minutes. If any gel slices were completely dry at that point, more TEABC mixture was added and slices were reincubated. Care was taken to standardize the amount of trypsin added between equivalent control and experimental slices. Once all gel pieces had been rehydrated with the trypsin mixture, they were incubated at 30°C overnight in the thermomixer. The following morning, the supernatant was collected

and dried in the SpeedVac. Gel extraction buffer was added to the gel slices and they were sonicated for 3 x 15 minutes in order to maximize peptide yield. The collected supernatant was added to the tubes with the dried peptides and again dried in the SpeedVac. Samples were then cleaned on C18 columns.

C18 clean up

In order to remove contaminants and errant gel particulates, peptides obtained from in-gel digestion were resuspended in 1% TFA and cleaned on a microspin C18 silica column (Nest group). All spins were done at low speed (~100 x g) on a tabletop centrifuge. Per manufacturer's instructions, columns were prequilibrated by washing three times with ACN. Samples were loaded onto the columns and then washed 3 times with 0.1% trifluoroacetic acid (TFA) in milliQ. Peptides were eluted with 0.1% TFA in 50% ACN then dried using a Savant SPD131 DDA SpeedVac Concentrator (Thermo).

Yeast peptide MS/MS and data analysis

For the yeast work, MS samples were prepared from silver stained gels and submitted to Van Kelly, Kshitiz Tyagi, and Patrick Pedrioli.Van Kelly was in charge of running and analysis of these samples while Kshitiz Tyagi and Patrick Pedrioli assisted with development of the approach, protocols, and general MS information. MS/MS was performed on an Orbitrap Velos ETD (Thermo). MS data was searched against SGD yeast protein database concatenated to a reversed sequence databased using XTandem! with the k-score plugin. Results were validated using the Trans Proteomic Pipeline (TPP) and proteins were filtered at a 1 percent false discovery rate. We then sorted the resulting hits based on strength and connection to the NEDD8 or ubiquitin pathways as well as possible connection to unique pathways.

Mouse testes peptide MS/MS and data analysis

Mammalian samples were prepared from Coomassie stained gels and submitted to David Campbell, facility manager of the Proteomics and Protein Chemistry Service of the University of Dundee. Mass spectrometric analysis was performed by LC-MS-MS using a Proxeon EASY-nLC system coupled to a linear ion trap-orbitrap hybrid mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (Thermo). Peptides were injected onto a Dionex (Part No.160321) Acclaim PepMap100 reverse phase C18 3µm column, 75µm x 15cm, with a flow of 300 nl/min and eluted with a 40 min linear gradient of 95% solvent A (2% Acetonitrile, 0.1% formic acid in H_2O) to 50% solvent B (90% acetonitrile, 0.08% formic acid in H_2O). The instrument was operated with the "lock mass" option to improve the mass accuracy of precursor ions and data were acquired in the data-dependent mode, automatically switching between MS and MS-MS acquisition. Data were analysed by searching the SwissProt/Mouse database using the Mascot search algorithm (http://www.matrixscience.com) on an in-house system.

Hits were filtered for peptide scores higher than 28 ($p<0.05$) indicative of identity or extensive homology. Common contaminants (keratin, BSA, IgG) were eliminated. The remaining hits were analyzed by searching for known pathway members. Connections between identified hits were also analyzed using both DAVID (http://www.david.abcc.ncifcrf.gov/home.jsp) and STRING (http://www.string-db.org/), publically available software for analyzing gene ontology (GO) terms. Pathways revealed by DAVID or STRING GO analysis and repeated high scoring hits were then

searched for on the Gene Ontology AmiGO (http://www.amigo.geneontology.org). The Gene Ontology AmiGO gives a list of reported interaction partners. The spectra matching our quality control parameters were then queried for the possible interaction partners identified using the Gene OntologyAmiGO.The reason for this added database search was to reveal possible pathway members that might not be recognized using the other search algorithms.

Chapter 3: Neddylation system regulation in *Saccharomyces cerevisiae.*

3.1 Introduction

Our initial aim was to identify NEDD8 substrates and regulatory mechanisms in *Saccharomyces* and subsequently confirm these findings in mammalian cells. We had planned to use overexpression as a preliminary technique for substrate identification. However, previous results have shown that such overexpression resulted in misactivation of Rub1 by the UAE and conjugation through the ubiquitylation system. We have therefore reported that NEDD8 overexpression results in atypical UAE dependent neddylation in both mammalian cells and yeast (Hjerpe et al., 2012a; Hjerpe et al., 2012b).

This observation opened up two unique problems. First, how was free NEDD8/Rub1 regulated to prevent atypical neddylation? We believe that the means by which free and conjugated NEDD8/Rub1 is regulated is central to resolving this question. Second, could we identify non-cullin proteins neddylated via NEDD8/Rub1 pathway enzymes at endogenous or near endogenous levels?

3.2 Selection of *Saccharomyces cerevisiae* as a model system

Baker's yeast has the unique distinction of being the only known eukaryote where the NEDD8 homolog, Rub1, is nonessential (Sela, 2012). This allows us to use Rub1 deletion strains that would not be viable in other organisms. Furthermore, many useful tools were available for use in such a project such as the yeast gene deletion library and straightforward genetic manipulation.

3.2 Rub1 degradation technique selection

In order to better understand how the two systems were insulated from one another, we decided to investigate the mechanism by which Rub1 was turned over. NEDD8 and ubiquitin share significant similarity. For this reason we theorized that there must be some specific means of degrading Rub1 and ubiquitin independently. Due to the similarities between their structure and sequence, similar techniques like radioactive pulse chase or mutation of the diglycine motif necessary for conjugation have been used to study their turnover (See section 1.3.8 Ubiquitin stability and turnover). However, unlike ubiquitin, turnover could not be studied using overexpression of conjugateable Rub1. This is because alterations in the ratio of NEDD8 to ubiquitin result in charging of the ubiquitin E1 with NEDD8 and ultimately atypical neddylation. Conversely, the NAE does not charge ubiquitin (Walden et al., 2003). We assumed that such aberrant regulation could result in abnormal degradation kinetics.

3.2.1 Tagged Rub1

Efforts to study the regulation of free Rub1 were hampered by the lack of an effective Rub1 antibody for use in WB and IP. Others in the lab had found that commercially available anti-Rub1 antibodies cross-react with ubiquitin. We were unable to visualize free Rub1 directly on WB. Initially we approached this problem by using tagged Rub1 while we developed an antibody.

3.2.2 Studying protein turnover in yeast

In yeast protein turnover is typically studied in one of a few ways: radiolabeled pulse chase assay (described in relation to ubiquitin, section 1.3.8.1), the cycloheximide chase

assay, and an overexpression chase assay. We made use of the second two methods in this project. Not only do such experiments allow us to examine Rub1 turnover, but we can also use them to test various strains. In strains lacking a component important for Rub1 turnover we would expect degradation to be stabilized. Therefore in identifying such strains we can identify components of the degradative pathway. These turnover assays can therefore be used to identify genes responsible for the regulation of Rub1 levels.

3.2.3 Cycloheximide chase assay

A cycloheximide chase can be and is used to determine the stability of a given protein over time by halting protein biosynthesis. Translation of the mRNA into protein by ribosomes is inhibited using the drug cycloheximide. Then the levels of the target protein are chased over time. The advantage to this technique is that the protein of interest does not need to be overexpressed. This technique has been used to effectively study ubiquitin turnover in yeast (Hanna et al., 2003).

The disadvantage of the cycloheximide chase assay is that it does not discriminate between the mRNA of interest and mRNA in general, but rather stops all ribosomal translation. This nonspecificity may generate many off target effects especially over longer time courses when proteins that are normally turned over are not re-synthesized. One measurable off target effect is the inhibition of the cell cycle. Growth of yeast after cycloheximide treatment stagnates. These off target effects are used to confirm that the cycloheximide treatment was effective as a growth curve or WB of quickly turned over proteins demonstrate the efficacy of ribosome inhibition. These side effects could also

indicatethe failure of regulatory mechanisms. This technique is therefore best used if substantiated with other methods. After obtaining a Rub1 antibody, we elected to use this technique to follow endogenous Rub1 turnover. Due to the possible off target effects we also placed this piece of evidence in context by performing several other experiments with untagged or endogenous Rub1.

3.2.4 Overexpression chase assay

The alternative to cycloheximide treatment used extensively in this project is the overexpression chase assy. This experimental set up is a sort of modified pulse chase where the pulse is overexpression of the protein of interest (in this case Rub1) followed by shut off of expression. As with a pulse chase, the presence of the protein in cell lysate is then chased over time. In these experiments, overexpression is regulated by an inducible promoter like the galactose inducible (GAL) promoter.

In yeast when a gene is under the control of the GAL promoter, that gene is only expressed if the most preferable energy source is galactose. Expression is low to nonexistent if that yeast is grown in media lacking galactose or if the media has the more preferred sugar, glucose. Using a GAL promoter for an overexpression chase assay is simple as this sugar-based regulation allows for controlled gene expression. (Hopper)

First, the strains of interest are transformed with plasmids carrying the construct of interest under the control of the GAL promoter. Then the yeast is grown in media

lacking galactose and glucose. Expression is induced by addition of galactose and halted by subsequent addition of glucose.

3.3 Advantages to the overexpression chase for Rub1

In order to pursue this technique with Rub1 we developed a variety of cDNA constructs in the pYES2 backbone, putting Rub1 variants under the control of the GAL1 promoter. These plasmids were transformed into various yeast strains and the overexpression chase assay was then run.

The advantage to this technique was that we could perform it immediately while we waited for the Rub1 antibody to become available. Another advantage to this approach is that when compared to the cycloheximide chase, this approach has fewer off target effects. The disadvantage to the overexpression chase is that it cannot function on endogenous levels of protein. Another problem is that overexpression of Rub1 results in crosstalk with the ubiquitin system. Furthermore we did not have an antibody against endogenous Rub1 so a tag was used until the Rub1 antibody became available.

3.4 Establishing the overexpression chase assay for Rub1

The Rub1 system posed several unique challenges for studying turnover. Since we did not initially have a direct antibody against Rub1, we used a tagged construct while we developed that an antibody. We elected to use overexpression so that we could clearly visualize and chase Rub1. A GAL1 promoter was used because of the control it gave us over overexpression. Since overexpression can force NEDD8 onto the UAE, we used a nonconjugateable construct. As with ubiquitin, mutation or deletion of the G75/76

diglycine motif abrogates conjugation of Rub1. We have tested both the deletion of this motif and the mutation to dialanine and have observed the same trends with both. When overexpressing conjugateable Rub1 we observed consistent clear accumulation of free Rub1 after one hour and accumulation of most UAE dependent Rub1-conjugates appear after that point. We therefore only overexpressed nonconjugateable Rub1 for 1 hour to minimize off target effects.

3.4.1 Tag selection

We made use of both single and triple N-terminal HA tags. We selected the HA tag as it is short (9 residues: YPYDVPDYA), unstructured, not targeted by the N-end rule, and would allow for easy detection and immunoprecipitation of HA-Rub1. We also attempted to use GFP tagged Rub1 but this was not efficient. Furthermore there would be questions over the viability of any data observed using such a tag, as it is approximately 3 times larger than NEDD8 itself. Nonetheless, two of the three yeast cullins are clearly neddylated by GFP-Rub1 following overnight overexpression in galactose containing media. Prior to my arrival in the lab HA-6xHIS-Rub1 was also utilized for work with endogenous Rub1 turnover and proteasome inhibition.

3.4.2 Plasmid selection

A galactose inducible promoter was selected as a means of specifically regulating Rub1 expression in initial screening while minimizing off target effects. Tagged Rub1 was inserted into the pYES2 vector. The pYES2 vector bears the 2µm origin of replication allowing for episomal maintenance of high plasmid copy numbers (10-40 copies/cell). This backbone also carries ampicillin resistance for selection of bacteria carrying the plasmid as well as the uracil biosynthesis gene URA3 for selection of transformed yeast

(2009). Overexpression of either 3HA-Rub1ΔGG or HA-Rub1 G75/76A was induced with galactose for one hour and then shut off of by addition of glucose.

3.4.3 Time course for overexpression chase assays

We followed a simple procedure to observe the degradation profile of 3HA-Rub1ΔGG or the dialanine equivalent. We overexpressed the protein for one hour. We chose a short expression time for two reasons. First it was to minimize artifacts as most overexpression conjugates observed using the conjugatable HA-Rub1 appear after that time and we reasoned that off target effects might be more likely following that time. Second we selected one hour because using the conjugateable form of HA-Rub1, free Rub1 is clearly and consistently visible after 1 hour of expression. We shut off expression by direct addition of glucose and collected samples at various time points for analysis of HA-Rub1 levels. Samples were prepared for SDS-PAGE using the TCA technique. Samples were run on SDS-PAGE then transferred from the resultant gel to a nitrocellulose membrane that was then used for WB. When a component of the degradation pathway is impeded, the degradation of the construct should also be impeded. In such an instance, HA-Rub1 is neither produced, because we have shut off expression, nor degraded, because we have eliminated an essential member of the degradation machinery. In such cases, the construct should appear to stabilize on WB.

3.5 Ubiquitin Proteasome System (UPS) mediated degradation

3.5.1 UPS dependence

Using this overexpression chase technique in wild type yeast reveals that nonconjugateable 3HA-Rub1ΔGG is degraded readily with significant decreases visible

by 15 minutes (Figure 3.1). This is in agreement with current opinion that NEDD8 is cleared quickly (Hipp et al., 2004).

3.5.2 Proteasome dependence

There are two main degradative pathways—the vacuole/lysosome and the proteasome. The yeast equivalent of the lysosome is known as the vacuole. Although when compared to the lysosome the vacuole has increased functions, such as storage, it still fills an important role in protein degradation (Armstrong, 2010). In yeast the main initiator peptidase of the vacuole is known as Pep4 (Liao et al., 2005). In a strain deleted for Pep4 degradation of 3HA-Rub1ΔGG is not impeded (Figure 3.1 A). This indicates that degradation is not proceeding in a vacuole dependent manner.

Inhibiting the proteasome provides a similar test case for UPS mediated degradation. The peptide aldehyde MG132 is a potent proteasome inhibitor (Liu et al., 2007). In order for yeast to take up this drug, the drug pump *pdr5*gene must first be deleted in the strains of interest (Liu et al., 2007). When strains lacking PDR5 were treated with MG132, the levels of 3HA-Rub1ΔGG appeared to stabilize on WB against HA (Figure 3.1 B). This indicates that upon inhibition of the proteasome degradation of 3HA-Rub1ΔGG was impaired.

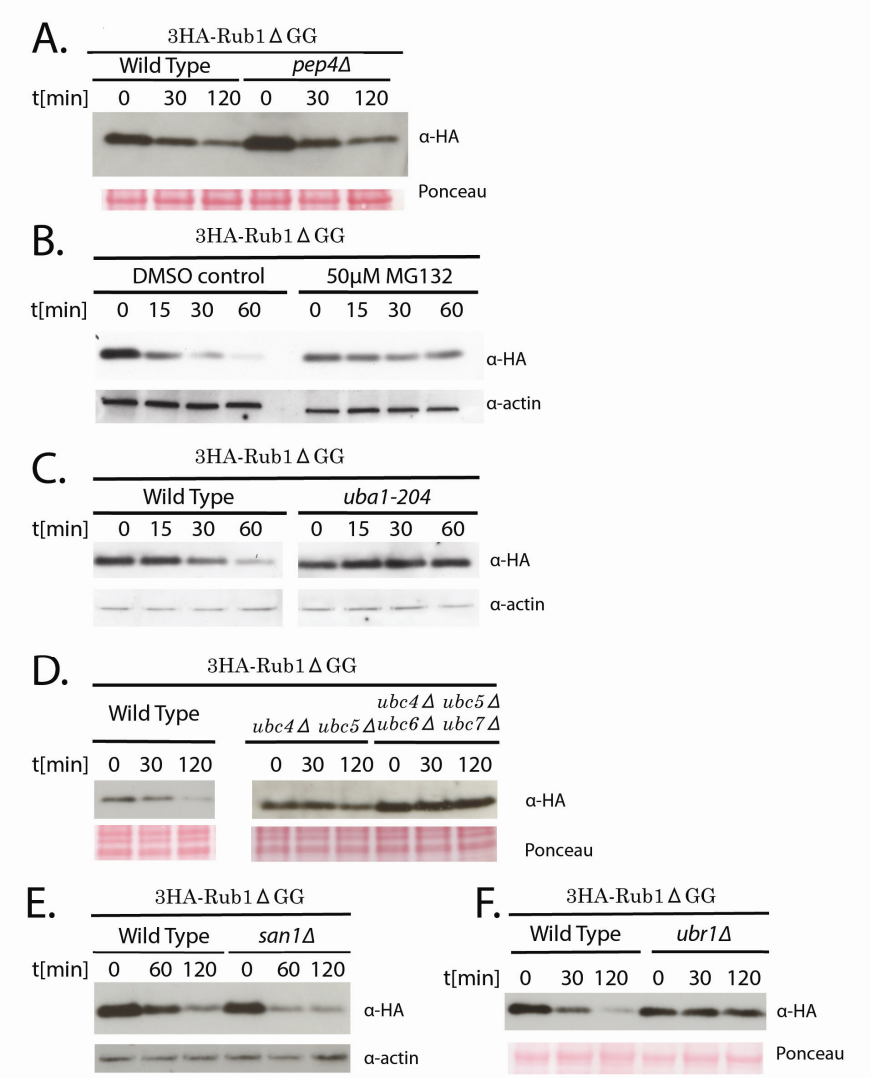


Figure 3.1 Degradation of 3HA-Rub1ΔGG is dependent upon the UPS and proceeds through the UPR. 3HA-Rub1ΔGG was overexpressed in the indicated strains for 1 hour. Expression was shut off and samples were taken at the indicated time points, TCA precipitated, resuspended in reducing sample buffer, run on polyacrylamide gels then Western blotted for HA. Stability indicates that the levels of the protein are stable even after expression is shut off. The strains where the HA signal is stabilized are impaired in degrading the construct.The tagged protein is not degraded via the lysosome as the *pep4Δ* strain does not have impaired clearance of the tagged construct (A). The proteasome inhibition (B) and *uba1-204* data suggesting that the protein is degraded in a proteasome and ubiquitin dependent manner was collected by Thimo Kurz prior to my arrival in the laboratory (C). The double deletion of *ubc4* and *ubc5* partially stabilizes the protein and the quadruple deletion strain enhances that phenotype (D). San1 (E) is not in the HA-Rub1ΔGG degradative pathway but Ubr1 is involved in clearance of the tagged protein (F).

3.5.3 Ubiquitin activating enzyme dependence

After proteasome dependence was established, we asked whether degradation was also ubiquitin dependent. Another UBL, FAT10, is degraded in a ubiquitin proteasome dependent manner (Buchsbaum et al., 2012) and we wanted to confirm if Rub1 was similarly degraded by ubiquitin. We believed this would be unique because of the high similarity between ubiquitin and Rub1. To test this hypothesis we reran the overexpression chase assay in a temperature sensitive (TS) UAE mutant (*uba1-204*). At the restrictive temperature, HA-Rub1 levels are stabilized in this mutant (Figure 3.1 C). These data suggest that HA-Rub1 ΔGG requires both the ubiquitin system and the proteasome in order to be degraded.

3.5.4 E2 identification

There are 13 E2 enzymes in yeast. We obtained deletion strains for 10 of them from our yeast KO collection and a TS mutant of the essential E2 *ubc3* (*cdc34-2)*. The two E2 deletion strains which were not included in this screen were *ubc1* and *ubc9*.The *ubc9*Δ strain was omitted partially because there was no deletion or mutation strain available in the laboratory. This omission is not believed to be significant because the gene is a Sumo specific E2 and does not participate with Rub1 or ubiquitin. The *ubc1* deletion strain was not in our deletion library or other laboratory collections and wastherefore not included in this screen.

The 11 E2 deletion/mutation strains were transformed with pYES2 3HA-Rub1ΔGG and the overexpression chase experiment was run. None of the E2 deletion strains had impaired clearance of 3HA-Rub1ΔGG.Similarly, at the restrictive temperature the *ubc3* TS mutant strain displayed no change in 3HA-Rub1ΔGG clearance compared to the

wild type. These findings indicate that no single E2 tested was associated with stabilization of 3HA-Rub1ΔGG.

There are some E2s, such as Ubc4 and Ubc5, which share significant similarity and are known act extremely promiscuously and to compensate for one another (Seufert and Jentsch, 1990). For this reason, a *ubc4/5* double deletion strain was included in this overexpression chase degradation panel. To a much lesser extent, Ubc6 and Ubc7 can compensate for the lack of Ubc4/5 (Chen et al., 1993) so the quadruple deletion strain was also included in this screen.

The multi-E2-deletion strains were transformed with the pYES2 3HA-Rub1ΔGG plasmid. The overexpression chase assay was run and presence of 3HA-Rub1ΔGG was tested for by WB against the HA tag. While the deletion of the individual E2 genes *ubc4, ubc5, ubc6, and ubc7* alone did not inhibit normal degradation of 3HA-Rub1ΔGG, the *ubc4/5* double deletion resulted in increased stabilization of the construct. The quadruple deletion strain (*ubc4/5/6/7 Δ)* increased the strength of the 3HA-Rub1ΔGG signal by WB (Figure3.1 D). Since the protein is stabilized, these enzymes are important in HA-Rub1 turnover. Degradation of 3HA-Rub1ΔGG requires one or all of these 4 E2 enzymes. As some compensation may occur only in cases where one gene has been deleted it is impossible to ascertain which enzyme or enzymes is primarily responsible for the degradation in vivo.

3.5.5 E3 identification

There are many known yeast E3s (54 in yeast, 1000 in humans (Staub and Rotin, 2006)). Due to the large number of known possible E3s, it was important to narrow the field of possible enzymes before running the degradation assay. We considered several approaches to this problem while working to identify the E2s involved in HA-Rub1 degradation.

There is some NMR and biochemical data on the structure of NEDD8. NEDD8 is almost topologically identical to ubiquitin but it spends more time in a semi-unfolded state and unfolds more easily than ubiquitin. Further, when exposed to proteases, like chymotrypsin,NEDD8 is degraded quite readily while ubiquitin is not fragmented. (Kitahara et al., 2006b).

There is very little that distinguishes the structures of Rub1/NEDD8 from ubiquitin and we postulated that a propensity to unfold could specifically target Rub1/NEDD8 for degradation while maintaining the integrity of the pool of free ubiquitin. We hypothesized that the UPR was responsible for selective Rub1 degradation. There are 2 UPR E3s in yeast—the nuclear San1 (Gardner et al., 2005) and the cytosolic Ubr1 (Eisele and Wolf, 2008). As we did with the possible E2s involved in degradation, we obtained *san1* and *ubr1* deletion strains from our KO library. These strains were transformed with pYES2 3HA-Rub1ΔGG. The overexpression chase experiment was run again and results were checked by WB against HA. Deletion of *san1* did nothing to abrogate HA-Rub1ΔGG turnover (Figure 3.1 E). This indicates that San1 is not likely involved in 3HA-Rub1ΔGG turnover. Deletion of *ubr1* resulted in clear stabilization of the HA-Rub1ΔGG construct (Figure 3.1 F). We further confirmed this finding by

transforming the *ubr1Δ* strain with HA-Rub1 G75/76A where there is only a single HA tag and the diglycine motif necessary for conjugation was mutated to nonfunctional dialanine rather than simply deleted. We again performed the overexpression chase assay and observed the same stabilization by WB.

Interestingly, Ubr1 can function in two pathways. First, N-end rule based degradation, requires the E2 Ubc2 (Kumar et al., 2010; Sadis et al., 1995; Xia et al., 2008). When working with the E2s Ubc4/5/6/7, Ubr1 is associated with the UPR (Eisele and Wolf, 2008). The stabilization of 3HA-Rub1ΔGG in the Ubr1 deletion strain, in conjunction with the stabilization of the same construct in the UPR associated E2 deletion strains *ubc4/5/6/7Δ* provides dual confirmation that degradation is going through the UPR. Furthermore, thisfinding is in agreement with our hypothesis that Rub1 might be distinguished from ubiquitin by some fundamental feature like low structural integrity.

3.6 Unfolded protein response mediated degradation might be artifactual

3.6.1 Degradation of near endogenous nonconjugatable Rub1

After identification of the overexpressed HA-Rub1ΔGG degradative pathway, we sought to confirm that UPR mediated degradation was also maintained at the endogenous level. In the absence of an effective Rub1 antibody we again relied upon tagging Rub1. Initially it was assumed that using a centromeric plasmid bearing the Rub1 promoter, two N-terminal tags (HA and His) and the *rub1* gene would generate near endogenous levels of Rub1. This is in line with common yeast practice. Free Rub1 does not accumulate sufficiently to be seen at WB levels (picograms of protein) in this experiment. However, we are able to see accumulation following proteasome inhibition

by MG132 (Figure 3.2 A). This finding indicated both that this protein was present in yeast extract at very low levels and that the proteasome was required for turnover of HA-His-Rub1ΔGG.

3.6.2 Endogenous expression of Rub1

As a control to ensure that expression of tagged Rub1 was at near endogenous levels, we decided to test cullin neddylation levels. We reasoned that if neddylation were similar to wild type we would have more confidence in our expression levels even without the ability to directly test Rub1 levels. We therefore expressed Rub1 on a centromeric plasmid and compared yeast bearing this plasmid to wild type yeast. It appeared by WB that Cdc53 neddylation was artificially low (Figure 3.2 B).

Including the 3'UTR as well as the Rub1 promoter on the centromeric plasmid returned cullin neddylation to normal levels (Figure 3.2 C). Addition of the 3'UTR also allowed us to observe free HA-Rub1 without proteasome inhibition. When we obtained a Rub1 antibody we were further able to confirm that the pRS413 Rub1 pr. Rub1 3'UTR and the equivalent HA tagged construct produce near normal free Rub1 levels. This finding demonstrates that the 3'UTR plays an important role in Rub1 expression that the Rub1 promoter (5' UTR) alone cannot compensate for. As it is nonstandard to include the 3'UTR when cloning yeast genes it is possible that this finding may be important for work on other genes. More testing will be needed to identify if other 3'UTRs similarly influence expression levels so greatly.

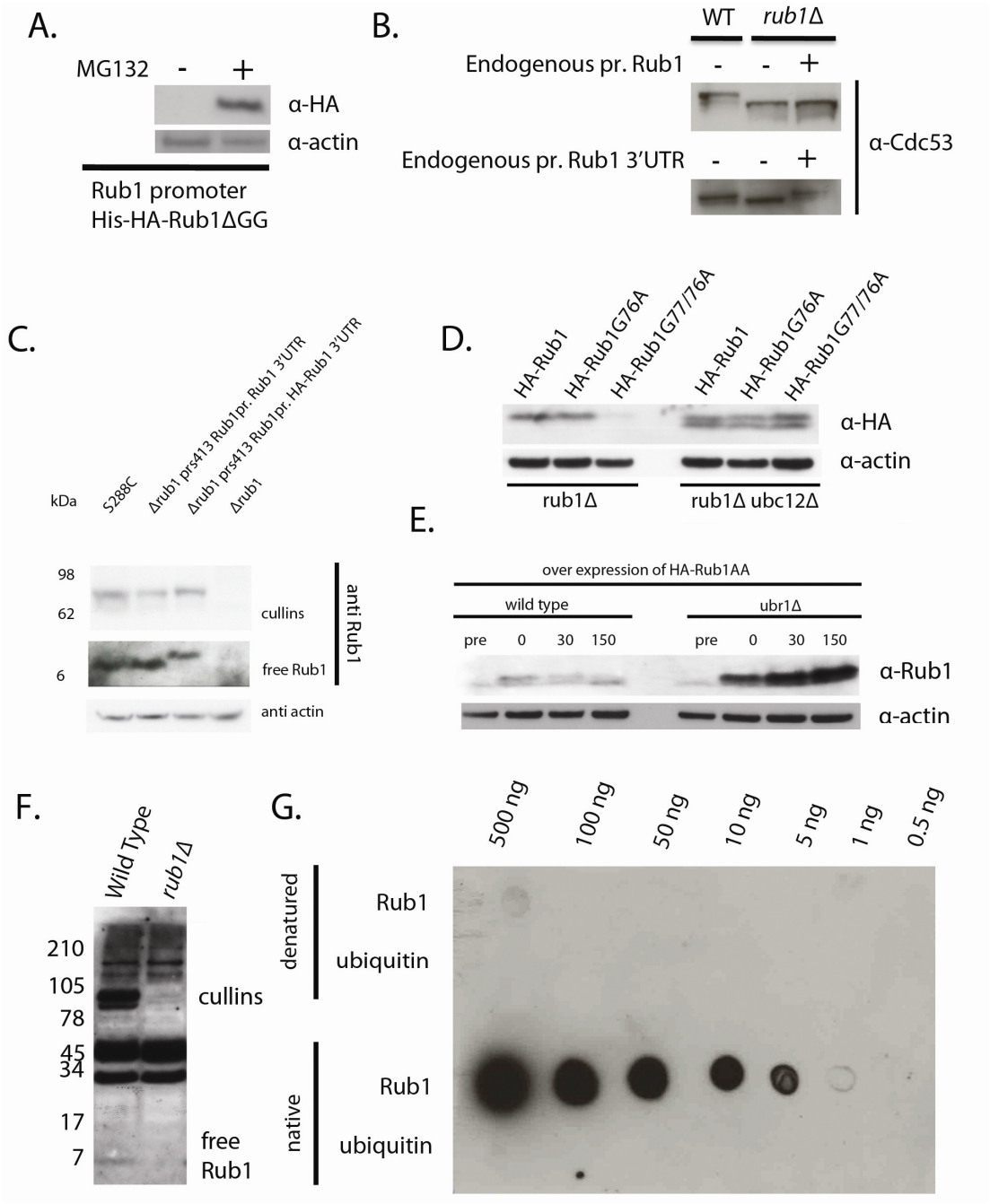


Figure 3.2. Regulation of near endogenous Rub1, tag dependence, and antibody characterization. Rub1 expressed under the endogenous promoter bearing His/HA tags accumulates only under conditions of proteasome inhibition (A). Expression of Rub1 requires the 5' and 3' UTRs for normal cullin neddylation levels (B). Rub1 under the control of the 3' and 5' UTRs expresses at near normal levels with and without tagging (C). A single HA tag on Rub1 is cleaved at near endogenous expression levels (D). Apparent degradation of tagged overexpressed Rub1 might have been due to tag cleavage (E). The Rub1 antibody specifically recognizes Rub1but has numerous nonspecific bands on WB (F) and does not recognize denatured Rub1 very strongly (G).

3.6.3 Diglycine mutation partially destabilizes Rub1

Once we had developed the correct settings to visualize HA-Rub1 by WB, we asked if the C-terminal mutations used to prevent conjugation were responsible for the observed instability and unfolding. Ubiquitin bearing a G76A mutation has been used in substrate studies because it interferes with the deconjugation by DUBS (Hodgins et al., 1992). However the ubiquitin needs to be mature—pro-ubiquitin bearing the G76A mutation does not become conjugated to substrates.

Similarly pro-Rub1 bearing a G76A mutation on a centromeric plasmid and expressed in a yeast background lacking endogenous Rub1 can be visualized by WB but is not effectively conjugated to the cullins. Interestingly, the level of free HA-Rub1 is fairly consistent between the G76A mutant and the wild type construct (Figure 3.2D). The G75/76A dialanine mutant on the other hand displays a marked decrease in free Rub1 when compared to both the single mutant and the wildtype (Figure 3.2 D). This data indicates that the dialanine or ΔGG mutations used to prevent conjugation could be a significant destabilizing factor in our degradation assays. This line of evidence suggests that free Rub1 is maintained at stable levels independent of its conjugation status but that the mutation of the diglycine motif in the short unstructured C-terminal region can be destabilizing.

3.6.4 Rub1 antibody development and testing

While working with both overexpression and tagging systems, we attempted to establish techniques for visualizing endogenous Rub1 directly. We initiated an antibody production campaign in sheep using an antigen from endogenous Rub1. We tested the antibody against purified ubiquitin and Rub1 (to test for cross reactivity) and against

WB of yeast with and without the *rub1* gene (Figure 3.2 F and G). The sheep anti-Rub1 antibody is specific for Rub1 but has many non-specific bands on WB that fall primarily between the cullins and free Rub1. These bands will impede the use of this antibody for substrate identification. Nonetheless, this antibody can be used to look at the cullins and free Rub1.

3.6.5 Untagged Rub1 is stable

Tagging can sometimes lead to aberrant regulation of tagged proteins (Hipp et al., 2004). We had already found that the diglycine mutation was somewhat destabilizing. We next needed to ask if the tags used contributed further the apparent quick UPR mediated degradation.

3.6.5.1 Untagged overexpression chase assay

First we ran the overexpression chase assay we had used before. The difference this time was that the construct we transformed into the yeast was untagged (pYES2 Rub1 G75/76A). This construct was overexpressed for one hour and then chased. Unlike the instability observed with the tagged construct, we observed apparent stability (Figure 3.2 E). This indicates that our earlier findings were likely artifactual.

3.6.5.2 Tag cleavage

We next asked whether tag cleavage could have contributed to the apparent degradation we had observed. If the HA tag was cleaved from HA-Rub1 G75/76A blotting for the HA tag might make the construct erroneously appear to have been degraded. WB of the tagged construct with a Rub1 antibody can be used to determine if this occurs because

the tagged form would migrate at a slightly higher MW than the untagged form. Therefore if a doublet is observed, there is likely tag cleavage also occurring.

To determine if this was possible we performed several experiments. First, we looked at an overexpression chase assay of the HA-tagged construct using the Rub1 antibody. It appears that the tagged form is reduced over time while the untagged form is unaffected or increased. This could mean the tagged form is less stable and therefore degraded (this is likely because of the much higher signal in the *ubr1Δ* background), the tag is being cleaved (this is also likely because the doublet is observed even in the *rub1Δ* background), or a combination of the two. In order to more clearly discern if the HA tag is cleaved we examined lysate of *rub1Δ* yeast carrying the pRS413 HA-Rub1 plasmid. There is clear evidence of a doublet on WB. This has been interpreted to mean that the HA tag is cleaved. This doublet is sometimes hard to discern but is stronger in strains where conjugation has been inhibited as with deletion of *ubc12*, the Rub1 E2 (Figure 3.2 D).

Although we did not expect a single HA tag to be cleaved, this is what we have observed. Such cleavage could have confounded some of our results as shifts from tagged to untagged Rub1 would in an anti-HA WB appear to be degradation (Figure 3.2 E). In fact, untagged overexpressed Rub1-AA is stable in the time courses using a galactose inducible construct (Figure 3.3 A). The stability observed here in conjunction with the tag cleavage and somewhat destabilizing role of the diglycine mutation all cast doubt on the relevance of the quick degradation observed with the tagged mutated Rub1.

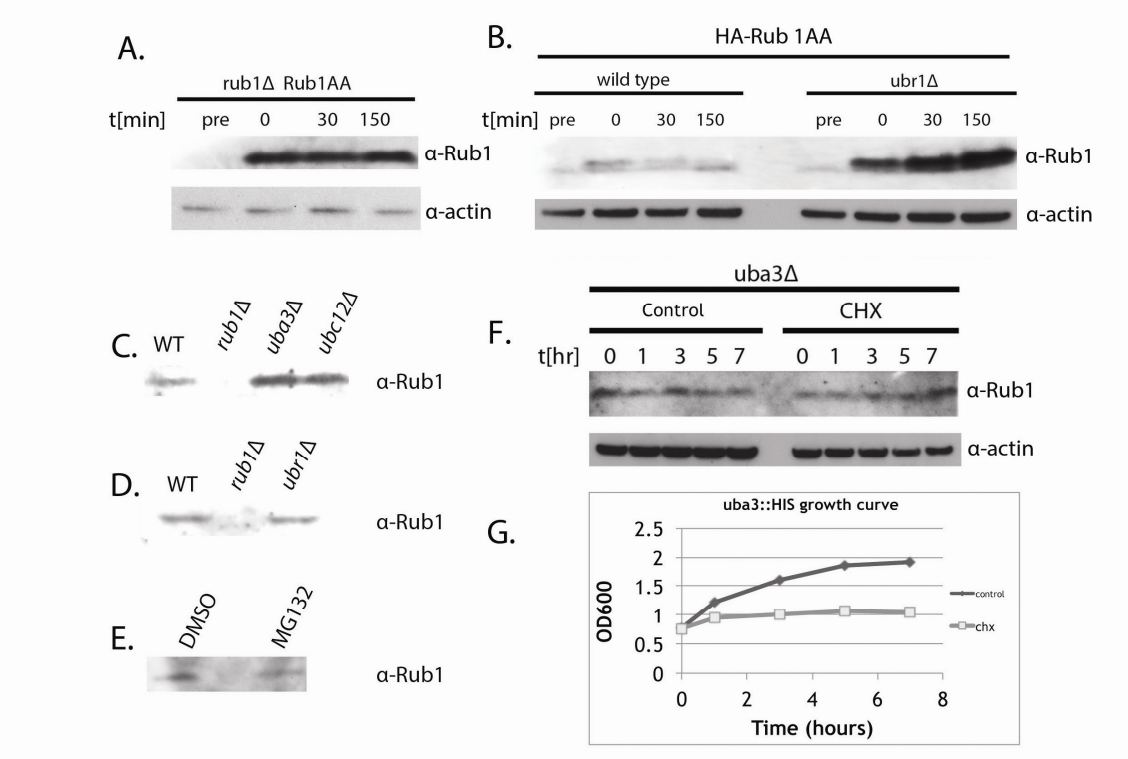


Figure 3.3 UPR mediated Rub1 degradation is artifactual and Rub1 is endogenously stable. Overexpressed Rub1 G75/76A is stable in the overexpression chase assay (A). Tag cleavage may contribute to apparent degradation of HA-Rub1 and expression of HA-Rub1 AA is stronger in a strain lacking *ubr1* (B). Deletion of the Rub1 pathway enzymes *uba3* and *ubc12* results in an increase in free Rub1 (C). Deletion of *ubr1* does not affect free Rub1 levels (D). Inhibition of the proteasome does not lead to dramatic changes in endogenous Rub1 levels (E). Free Rub1 levels are stable over 7 hours after inhibition of the ribosomes by cycloheximide treatment (F). The growth curve of the samples from cycloheximide chase in F, confirms that the cycloheximide treatment was effective (G).

3.6.7 Endogenously expressed Rub1 does not go through the UPR

In strains lacking either the NAE (*uba3*) or the rub1 E2 (*ubc12*) conjugation via the Rub1 pathway is abrogated and the entire pool of Rub1 should remain free. Interestingly, in strains deleted for these genes, we observed an increase of endogenous untagged Rub1 when compared to wild type yeast (Figure 3.3 C). One possible explanation for this observation is that the Rub1, now in the free pool, would typically

be conjugated to substrates such as the cullins. If turnover was quick or closely controlled we would not expect such an accumulation.

3.6.7.1 Ubr1 is not important for maintaining Rub1 levels

Unlike the accumulation observed in the Rub1 pathway deletion strains, deletion of *ubr1* does not lead to an accumulation of Rub1 (Figure 3.3 D). If Ubr1 mediated Rub1 turnover endogenously, a similar backlog of free Rub1 might be expected to accumulate. There are near WT levels of free rub1 observed in WB of *ubr1Δ* strains. Therefore degradation of Rub1 is unlikely to endogenously proceed through this E3.

3.6.7.2 Proteasome inhibition does not stabilize Rub1

We further wanted to ask if Rub1 was even degraded by the proteasome. To answer this question, we grew yeast expressing Rub1 from the endogenous locus and then treated it either with the proteasome inhibitor MG132 or the equivalent volume of vehicle (DMSO). We then checked the level of free Rub1 by WB. Proteasome inhibition does not result in an accumulation of Rub1 (Figure 3.3 D). This data suggests that Rub1 is not turned over in a Ubr1/proteasome dependent manner.

3.6.8 Rub1 is a stable protein

Since untagged Rub1 does not appear to be degraded in a Ubr1 or proteasome-dependent manner, we decided to use a cycloheximide chase to follow endogenously expressed Rub1 turnover. For this experiment, we selected a *uba3Δ* strain. By deleting the NAE, we hoped to abrogate conjugation through the neddylation machinery much like we did with the diglycine mutation in earlier experiments. In this way we do not need to worry about the possibly destabilizing diglycine mutations but can ensure that

we are following a free pool of Rub1. Without overexpression we don't expect significant crosstalk with the ubiquitin pathway.

In this experiment, we shut off expression of Rub1 by cycloheximide treatment and then collected samples for 7 hours (Figure 3.3 F). We used a growth curve to confirm that the cycloheximide treatment was working(Figure 3.3 G). We observed by WB that Rub1 levels remain stable for all 7 hours. This finding contrasts with the degradation of the HA-tagged overexpressed construct visible by WB after only 15 minutes. Endogenous free Rub1 is much more stable than when tagged and mutated.

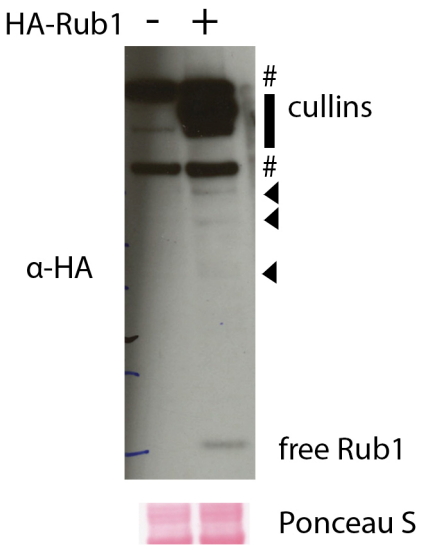


Figure 3.4.Possible HA-Rub1 substrates. In the presence of HA-Rub1, the cullins and free Rub1 are visible by WB. There are nonspecific antibody reactive bands (denoted by #). Some apparent non-cullin HA-Rub1 bands are also visible by WB (denoted by black triangles). These may represent genuine Rub1 substrates.

3.7Substrate identification in yeast

Although we were unable to determine the means by which free Rub1 was degraded, we thought we might still be able to discern which proteins were conjugated to Rub1 endogneously (Figure 3.4).

3.7.1Tagged Rub1

In our investigations of yeast strains harbouring the near endogenous HA-Rub1 plasmid, we observed HA-reactive bands on WB that did not exactly match the MW of the yeast cullins (Figure 3.4). Although weak and

inconsistent, these bands could be interpreted as low abundant non-cullin substrates so we attempted to identify them.

3.7.2Untagged Rub1

Ideally, identification of substrates is undertaken with no changes to endogenous expression. Therefore we attempted to use the anti-Rub1 antibody generated for this project. This antibody generates many nonspecific bands in the MW region below the cullins and above free Rub1 (Figure 3.2F), which, coincidently, was where we had seen the putative Rub1 substrates (Figure 3.4). These nonspecific bands prevent clear analysis of endogenous substrates via WB. We therefore utilized two techniques meant to enhance substrate visibility. First we attempted to IP neddylated proteins using the Rub1 antibody. However attempts to do this were unsuccessful. This might bear revisiting because it was not extensively tested.

Second, we screened DUB deletion strains for aberrant levels of free Rub1, cullin neddylation, and abnormal banding patterns. For this screen we also included a strain lacking *rfu1* (a gene encoding negative regulator of the DUB Ubp4). All DUB deletion strains are viable and available from our yeast KO collection. Strains were grown and harvested in complete YPAD media under non-stressed conditions. The WBs of the knockout strains were probed with anti- Rub1. Most DUB deletion strains did not display significant differences from the wild type strain (Figure 3.5A).

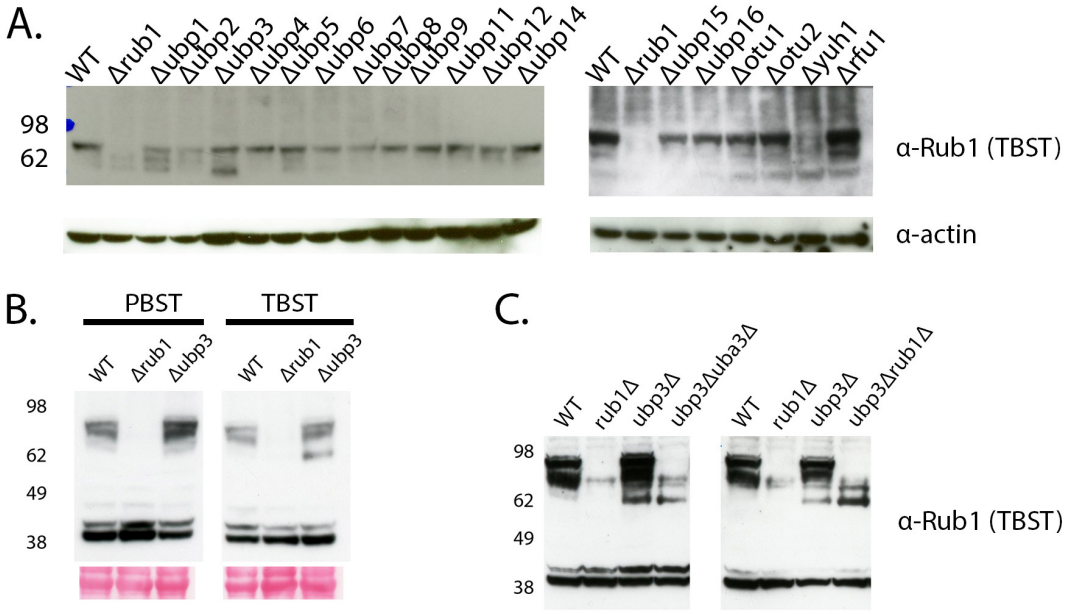


Figure 3.5. DUB deletion as a means of Rub1 conjugate identification. A series of yeast DUB deletion strains blotted for Rub1. There are no strongly enriched bands when compared to wild type except in the *ubp3*Δ strain (A). The *ubp3*Δ phenotype is stronger when the WB is developed in TBST based buffers (B) and is not dependent upon the NAE (Uba3) or Rub1 itself (C).

3.7.3Ubiquitin specific protease 3 (Ubp3)

There was only one striking new antibody reactive band on WB significantly enriched by deletion of a DUB. Following the deletion *ubp3,* a rub1 antibody reactive band of about ~62kD is strengthened. This band was most striking when the wash buffer was TBST rather than PBST (Figure 3.5B). While some antibodies function better in one wash buffer than the other, such behavior may indicate that the band is nonspecific. We wanted to unequivocally determine whether that was the case. Towards that end, two double deletion strains were generated.

A strain lacking *uba3* cannot activate Rub1 or conjugate it to substrates. If the band of interest were genuinely conjugated to Rub1 through the neddylation machinery, the

observed enrichment of the band of interest would be reduced or eliminated by such a deletion. However, this phenotype was not rescued by deletion of the NAE *uba3*. WB of the *ubp3Δ strain* looked the same as the *ubp3Δuba3Δ* strain (Figure 3.5C). The~62 kD band was not neither reduced nor eliminated by NAE deletion and therefore is accumulating independent of the neddylation pathway.

Rub1 may become conjugated through the ubiquitin pathway rather than in a NAE dependent manner. Therefore in order to absolutely determine if the ~62 kD band represented a truly nonspecific target of the anti-Rub1 antibody, the *rub1* gene was deleted in a strain already bearing the *ubp3* deletion. In such a strain there can be no contribution from Rub1 to the protein pool and therefore any antibody reactivity observed is nonspecific. The *ubp3Δrub1Δ* strain was generated. We observe by WB that the enriched band is unaffected in the double deletion when compared with the *ubp3* deletion alone (Figure 3.5C). This observation in, conjunction with the results of the *uba3Δubp3Δ* double deletion WB, proves that this band is purely nonspecific and not dependent upon the neddylation pathway.

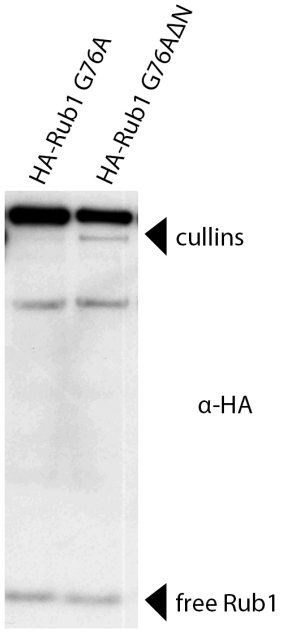


Figure 3.6. Mutation of the terminal glycine does not enrich for Rub1 substrates.

3.7.4Tagged Rub1 αNEDD8

3.7.1 Mutation of the terminal glycine

The first two approaches to enriching Rub1 conjugates were unsuccessful so alternative

means were investigated. Mature ubiquitin bearing a G76A mutation is a reported means of identifying some low abundant ubiquitin substrates (Geng and Tansey, 2008; Hodgins et al., 1992). We tested if this method would be applicable to neddylation as well. In order to test if this method would enrich for neddylation, we transformed a *rub1Δ* strain with pRS413 Rub1 pr. HA-Rub1 G76A ΔN77 3'UTR. We observed that mature HA-Rub1 G76A can only inefficiently conjugate to cullins and does not reveal new substrates by WB (Figure 3.6).

3.7.2 Immunoprecipitation of HA-Rub1

As we were unable to artificially pre-enrich for substrates or immunoprecipitate untagged Rub1, we initiated a large-scale immunoprecipitation program in order to enrich HA-Rub1 conjugates. The strains selected for this procedure were *rub1Δ* and *rub1Δubc12Δ* both carrying plasmids expressing HA-Rub1 at near endogenous levels. These strains were selected because there would be no competing endogenous Rub1. Furthermore, the control strain could carry the exact same plasmid as the experimental strain but would be unable to conjugate the construct to substrate proteins as it lacks the requisite E2 enzyme (Ubc12).

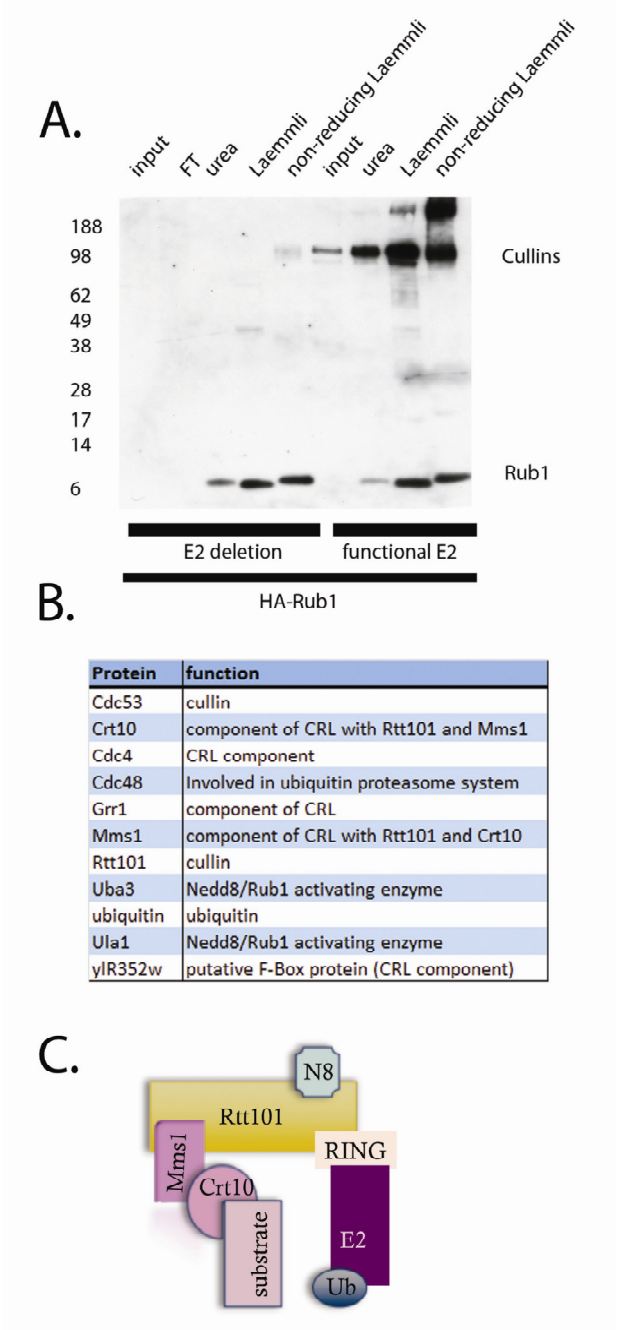
While a gel-free approach can often give more information about specific proteins pulled down by IP, the relative abundance of Rub1 and the neddylated cullins compared to other putative substrates was cause for concern. We believed that a gel-free approach would result in overwhelming low abundant substrates with peptides from Rub1 and cullins. We reasoned that by separating proteins by size via SDS-PAGE we could select specific bands of interest at various molecular weights and minimize the probability of

losing low abundant substrates under the noise of the cullins. Gels were silver stained and prepared for MS analysis.

3.7.3 Mass Spectrometric analysis of Rub1 associated proteins

Despite the success of the HA-IP (Figure 3.7A), there were no new substrates identified. Ultimately, the only peptides identified following this IP procedure were components of CRL complexes (Figure 3.7B and C). It should be noted here that only two of the three yeast cullins were identified. Cul3 was not found in the IP. This is consistent with the work of Aleksandra Zemla in our lab who has observed that Cul3 neddylation is low.

Given the possibility that the HA tag was cleaved in these strains we cannot rule out the possibility that Rub1 does have non-cullin substrates. Also the lack of Cul3 identification could indicate that our coverage of the Rub1 associated proteome was not high enough to identify low abundant substrates. We believe the strongest possibility is that any putative substrates seen were cullin degradation products. This analysis would account for the inconsistencies in our ability to visualize the bands of interest on WB.



Protein	function
Cdc53	cullin
Crt10	component of CRL with Rtt101 and Mms1
Cdc4	CRL component
Cdc48	Involved in ubiquitin proteasome system
Grr1	component of CRL
Mms1	component of CRL with Rtt101 and Crt10
Rtt101	cullin
Uba3	Nedd8/Rub1 activating enzyme
ubiquitin	ubiquitin
Ula1	Nedd8/Rub1 activating enzyme
ylR352w	putative F-Box protein (CRL component)

Figure 3.7. Rub1 substrate identification. Native HA-Rub1 IP from strains with and without the Rub1 E2, Ubc12. 5% of the IP was loaded on a 4-12% Bis-Tris polyacrylamide gel for WB (A) and the remaining 95% loaded on a 10% polyacrylamide gel, silver stained, and analyzed by mass spectrometry. Top hits from the MS analysis are listed (B). The Mms1 complex was identified very strongly. This cullin based complex (C) may have come down with Rtt101 and scored highly because Mms1 is a very large protein capable of producing many peptides.

4: Mammalian NEDD8

4.1 Introduction

The evidence given in chapter 3 suggests that neddylation in yeast may be limited to cullins. We hypothesized that in more complex organisms, with more complex NEDD8 specific regulatory mechanisms, neddylated proteins might be more abundant or more easily identified. NEDP1, for example, is a highly specific deneddylase with no yeast homolog (Mendoza et al., 2003). The existence of such a deneddylase suggests there may be non-cullin substrates regulated by neddylation and deneddylation. Alternatively it could suggest that NEDP1 exists to edit proteins neddylated via atypical activation in the ubiquitin pathway. Given the existence of NEDP1 in mammalian cells, we decided to update our model organism from yeast to human and mouse cells. We again asked whether we could identify non-cullin substrates of the neddylation pathway.

4.2 Neddylation in cultured human cells

First, we wanted to ensure that we selected a cell line where endogenous NEDD8 expression was visible by WB. We tested both the human osteosarcoma cell line U2OS and the human colon carcinoma cell lineHCT116. We observe apparent non-cullin bands (primarily below the molecular weights of the cullins) in both cell lines. In order to ascertain if these were genuine substrates of the neddylation cascade, we inhibited the NAE using MLN4924 (Figure 4.1 A). There was a significant decrease in apparent substrates following this treatment supporting the hypothesis that these substances were neddylated via the neddylation pathway.

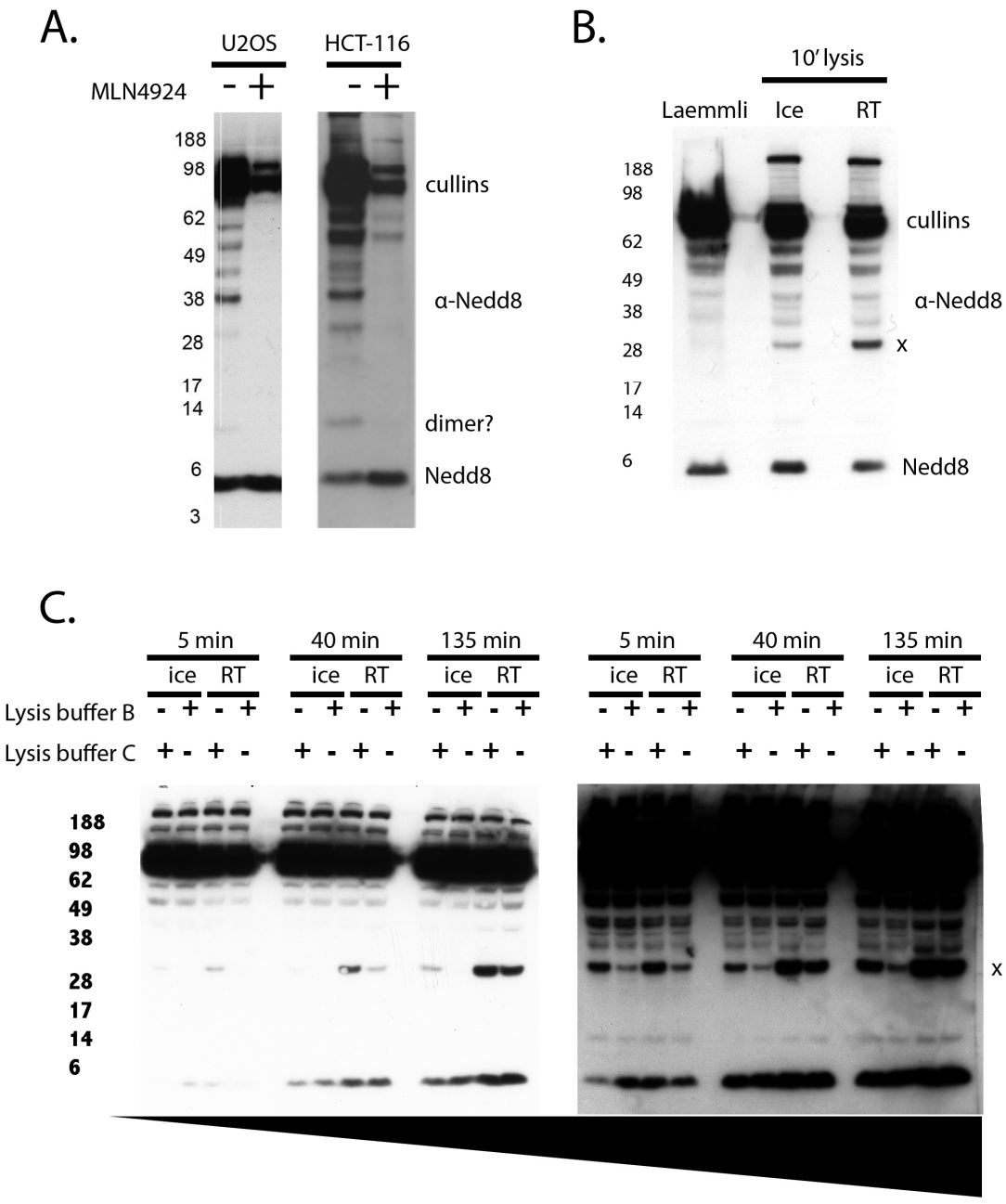


Figure 4.1. NEDD8 conjugates in cultured cells. NEDD8 conjugates depend upon the NAE in HCT116 and U20S cells (A). Lysis in reducing sample buffer (Laemmli) or in lysis buffer A on ice or at RT (B) reveals some putative substrates depend upon the lysis buffer stringency. Lysis under two different buffers (Lysis buffer B containing complete mini EDTA free protease inhibitoror Lysis buffer C containing complete protease inhibitor) over different times and different temperatures (C). The strong degradation product is denoted by x.

4.3 Testing for degradation products

Most apparent substrates appear below the molecular weight of the cullins on WB. In order to rule out the possibility that these NEDD8 reactive species were cullin degradation products, samples were prepared under various lysis conditions. In all of these related experiments, cells were harvested by trypsinization, washed with ice cold PBS and then split into eppendorf tubes for final lysis in various different buffers. Neddylated proteins that are merely products of proteolytic cleavage of genuine substrates should display an increase in relative abundance under conditions where proteases most active such as buffers with reduced protease inhibitors or when lysed at higher temperatures. Putative substrates that remain consistent under such conditions might represent genuine non-cullin conjugates because their formation is not dependent upon the activity of proteases following cell lysis.

The first set of lysis conditions revealed a noticeable difference in the neddylation profile as viewed by WB (Figure 4.1 B). In order of decreasing stringency these conditions were lysis directly in laemmli buffer or lysis buffer A on ice or at room temperature for 10 minutes. The strength of the strongest band not consistent with the MW of the cullins (~30kD) increases when proteolytic activity is highest (denoted by an x in Figure 4.1 B). For example, in lysis buffer on ice for 10 minutes, the band is much less pronounced than when allowed to lyse at room temperature where proteases are expected to be more active. Furthermore, the band is almost indiscernible when cells are lysed in Laemmli buffer directly. Laemmli buffer is the harshest buffer in this panel as it contains 2% SDS which can inactivate proteases via denaturation. This phenotype likely indicates that this anti-NEDD8 reactive band represents a degradation product

rather than a unique substrate. There are some NEDD8 reactive species falling between 38-62kD that remain consistent under various lysis conditions so these bands may represent NEDD8 substrates.

To confirm this observation, lysis was performed using two temperatures, three time points, and two different lysis buffers (B and C). Longer and warmer lysis times are more likely to allow for degradation products to form. The difference between the two lysis buffers was the type of protease inhibitor used. Lysis buffer C containing a 5X excess of Complete Protease Inhibitor Tablets (including EDTA) from Roche should more effectively inhibit degradation than lysis buffer B with Complete Mini-EDTA Free Protease Inhibitor Tablets from Roche. Cells were lysed either on ice or at room temperature for 5, 40, or 135 minutes before clarification at high speed and mixed 50/50 with Laemmli buffer (Figure 4.1C).

The major band observed in the first lysis buffer panel experiment (denoted by an x in Figure 4.1 B and C) increased conversely with buffer stringency. With respect to that major band, similar behavior is observed here. It is least prevalent in the lysis buffer containing extra protease inhibitors including EDTA and the longer the sample is allowed to lyse, the more pronounced the band becomes. In addition, the level of free NEDD8 rises over time confirming that proteolysis is occurring and freeing NEDD8 from substrates. The last observation of note from this experiment is that, as with the previous experiment, there are potentially genuine substrates where anti-NEDD8 WB band intensity does not appreciably change under any of the conditions tested

specifically between 49 and 62 kD. These species will require purification in order to confirm that they are not cullin degradation products.

4.4 Deneddylases

There were two compelling reasons to evaluate the effect deneddylase knockdown would have on apparent NEDD8 conjugation by WB. First, we wanted to determine if the anti-NEDD8 reactive bands between 49 and 62 kD were likely to be degradation products. CSN5 is believed to function solely as a cullin deneddylase. If the bands of interest are affected by CSN5 knockdown, that is strong evidence that they are cullin degradation products. If, however, levels are CSN5 independent they are more likely to be non-cullin substrates. Second, by knocking down deneddylases we can inhibit deconjugation of NEDD8 from substrates thereby enriching our proteins of interest. If deneddylase knockdown provides a means to enrich substrates and enhance our ability to detect and identify them, such a method could be used in conjunction with purification techniques to isolate these substrates. Consequently, all three definitive deneddylases CSN5, NEDP1, and UCHL3 were knocked down using siRNA.

4.4.1 CSN5

Following knockdown of CSN5 there was a moderate NAE1 dependent enrichment for bands around 49-62 kD (Figure 4.C) but other NEDD8 reactive bands were unaltered. While certain bands, such as those between 49-62 may represent cullin degradation products this is not confirmed. There are still some possible substrates that are consistent between the CSN5 siRNA and control siRNA treatments (especially at ~55 kD). Furthermore, the strong enrichment for NEDD8 reactive species observed

with NEDP1 siRNA is not observed here so all NEDD8 conjugates are not necessarily cullin degradation products. It is possible that some low abundant substrates are simply not visible in these WBs.

4.4.2 NEDP1

When the highly specific (and fairly promiscuous) deneddylase NEDP1 was knocked down, a multitude of NAE dependent bands in WB against NEDD8 appear or are strengthened (Figure 4.2 B). There are several possible explanations for the observed enrichment. First, NEDP1 could be deneddylating endogenous non-cullin substrates. This possible function would prove very useful in our exploratory efforts. NEDP1 knockdown may be a tool that can be used in conjunction with other purification techniques to identify neddylated proteins from cultured cells. Second, NEDP1 could be deneddylating cullin fragments. Such a function could be important for keeping NEDD8 from being turned over along with the cullins. If this is the appropriate function of NEDP1, then most if not all of the putative substrates observed on WB are cullin degradation products. One final possibility which we considered here was that NEDP1 plays a smaller role in vivo but during our lysis procedure NEDP1 is not effectively inhibited and therefore deneddylates normally neddylated proteins in solution in a non-physiologically relevant way.

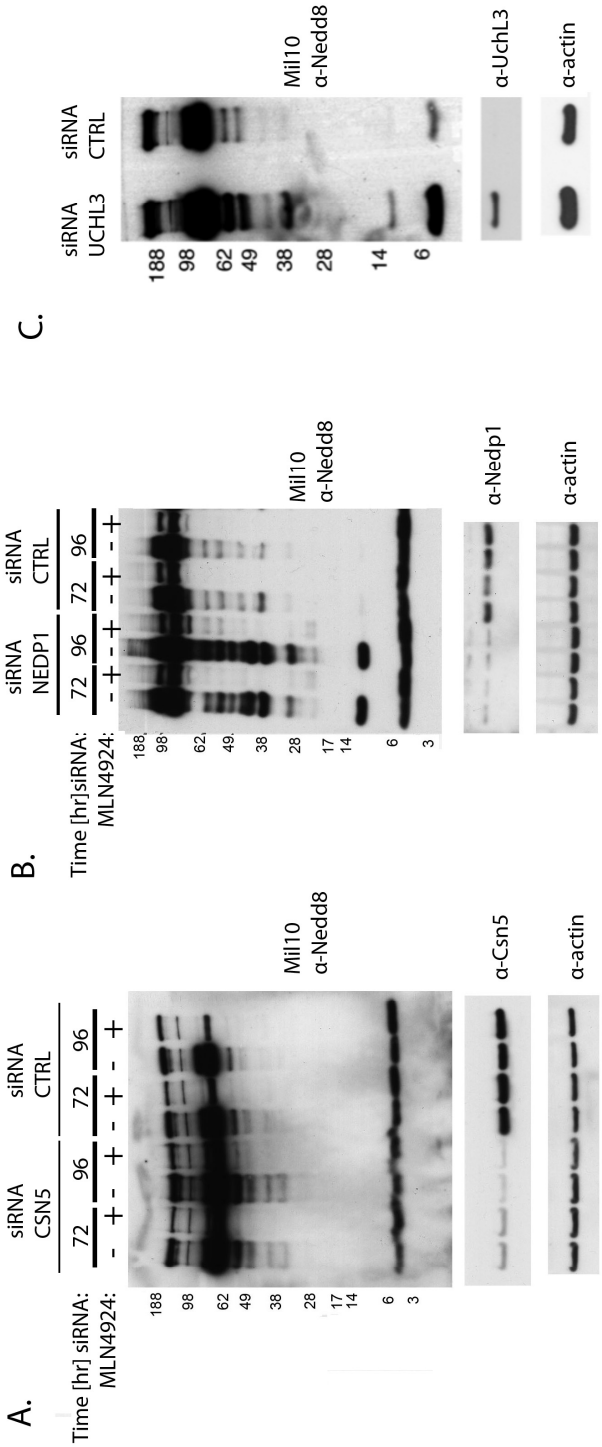


Figure 4.2.Deneddylase knockdown by siRNA in U2OS cells. CSN5 knockdown partially increases neddylation in an NAE dependent manner when compared to control (A). NEDP1 knockdown dramatically increases the neddylation profile in U2OS cells in an NAE dependent manner (B). UCHL3 knockdown in U2OS decreases apparent neddylation and free NEDD8 when compared to control siRNA (C).

The good enrichment for neddylated bands following NEDP1 siRNA that do not correspond to the MW of the cullins offers potential for future research. Knockdowns of NEDP1 may be a useful tool in identification of noncullin substrates. Such work may also help illuminate the poorly understood role of endogenous NEDP1 in vivo. A NEDP1 knock-in cell line is currently being developed in the laboratory and tested to determine exactly what that role is. Until such a cell line is tested it is hard to prove what functions NEDP1 is performing in vivo or in the lysis buffer.

4.4.3 UCHL3

UCHL3 is responsible for, among other things, the maturation of pro-NEDD8 to NEDD8 by cleavage of a 5 amino acid C-terminal extension (Hemelaar et al., 2004). It is possible that NEDP1 compensates somewhat for this role of UCHL3(Wu et al., 2003). However major contributions to Neddd8 maturation come from UCHL3. We hypothesized that if NEDD8 were not matured properly following UCHL3 knockdown, NEDD8 would not be conjugated to substrates effectively. This is exactly the phenotype we observed following UCHL3 knockdown. UchlL3 siRNA treatment results in widespread reduction in the appearance of NEDD8 reactive species and a concurrent reduction in free NEDD8 (Figure 4.2 C).

There are other conclusions from the UCHL3 knockdown experiment that have consequences that are more immediately clear. On the level of whole organisms, the UCHL3 dependent regulation of neddylation could have health implications. In brief, problems with global neddylation levels could result in some predictable deficiencies

with CRLs. Furthermore, some conditions such as AD display decreased UCHL3 activity in affected areas of the brain (Dennissen et al., 2011). Perhaps decreased activity of UCHL3 effectively functions like an in vivo knockdown of UCHL3 resulting in defects in neddylation like that observed following UCHL3 siRNA treatment. Neddylation defects could then contribute to some observed phenotypes, like inhibition of the UPS, in these instances. This vein of thought inspired a change of focus to UCHL3 related phenotypes which we then followed up.

Another phenotype observed following UCHL3 knockdown is the decreased growth of cells, which was clearly visible during routine checks of UCHL3 knockdown plates. This observation was followed up with Fluorescence Activated Cell Scanning (FACS) analysis. It should be noted before any analysis, that the FACS must be repeated and the siRNA deconvoluted as it is comprised of a pool of 4 oligomers. The FACs experiment was done in duplicate on one day and should be done on an independent day to minimize potential experimental bias if it is to be strongly considered. That being said; we know that UCHL3 itself is being knocked down as we have an antibody against UCHL3 and have tested the knockdown via WB. FACS analysis reveals that the phenotype observed by eye was the result of significant cell cycle arrest. There is approximately a 32.7% increase in the number of cells in G1, and decreases in the percentage of cells in both S and G2/M (27.9% and 5.9% respectively) (Figure 4.3).

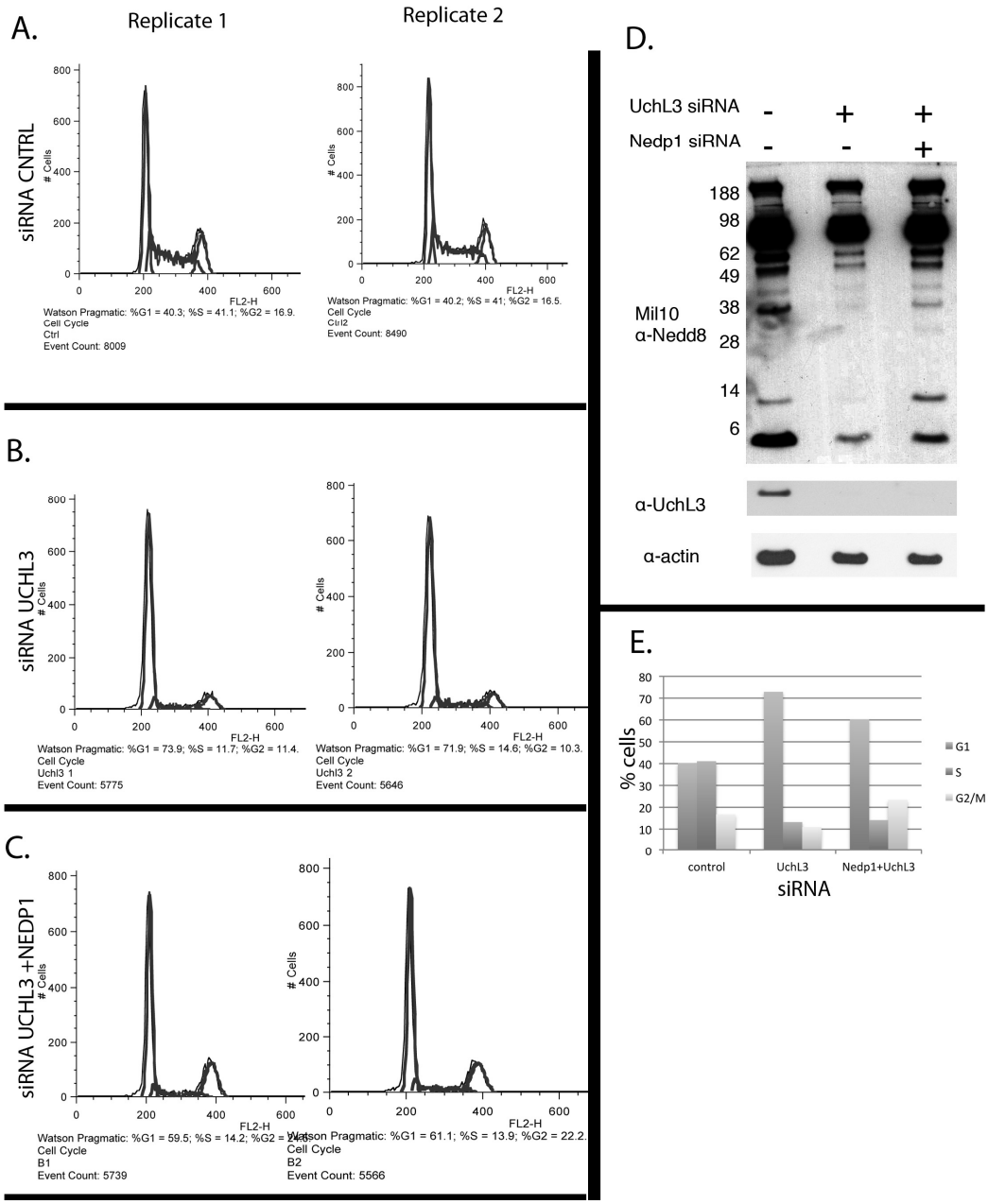


Figure 4.3.FACS analysis of cells following deneddylase knockdown. FACS profiles following transfection with control siRNA (A), UCHL3 siRNA (B), and cotransfection with UCHL3 and NEDP1 (C). WB showing siRNA phenotype. There is no Nedp1 WB because there was no available NEDP1 antibody when this experiment was performed (D). The cell cycle profiles of the three different treatments compared by percent in each phase (E)

We reasoned that if NEDP1 knockdown increased apparent NEDD8 conjugation and UCHL3 knockdown decreased such conjugation, knockdown of one might be able to rescue the other. Although NEDP1 knockdown alone was not included in this test, cotransfection of siRNA against NEDP1 and UCHL3 partially rescued the phenotype caused by UCHL3 knockdown both with regards to neddylation on WB and cell cycle (Figure 4.3 D and E). This work needs to be repeated on an independent day if it is to be used as the basis for future work. The knockdown of NEDP1 alone would also need to be included in the panel of siRNA conditions tested. The important take away message we want to draw from these data is not the observed cell cycle defects themselves but rather the possible importance of a UCHL3 and NEDD8 disease connection. This possibility led us to reevaluate the currently available literature.

4.5 Mammalian tissues

The observation of the cell cycle and neddylation defects in the UCHL3 knock down experiments, and the recent reports that UCHL3 function may be impaired in affected areas of Alzheimer's Disease brains (Dennissen et al., 2011), convinced us to review the pertinent literature. The UCHL3 knockout mouse displays growth retardation (Semenova et al., 2003), and certain tissue specific phenotypes but the ubiquitylation profile appears consistent between WT and knockout mice by WB of tissue lysates (Setsuie et al., 2009a). There are clear phenotypes in distinct tissues, specifically adipose tissue (Setsuie et al., 2009b), skeletal muscle (Semenova et al., 2003; Setsuie et al., 2009b; Setsuie et al., 2010), photoreceptors of the retina (Sano et al., 2006; Semenova et al., 2003), and the testis (Kwon et al., 2004). Knockout mice also have been reported to have short-term memory loss and abnormalities of the brain (Kurihara

et al., 2001; Wood et al., 2005). It is unclear whether it is the failure of UCHL3 as a deneddylase, a deubiquitylase, or a combination of the two that results in these specific deficiencies. Tissues of the UCHL3 KO mouse have been compared to WT tissues by anti-ubiquitin WB. I am unaware of a similar NEDD8 focused experiment. We therefore posited that there might be tissue specific neddylation that could account for the tissue specific phenotypes of the UCHL3 KO mouse. TheUCHL3 mouse data could hint that neddylation is tissue specific and by working with cancer cell lines we might miss some obvious substrates.

4.5.1 Tissue Panels

In order to confirm whether there were tissue-specific neddylated proteins we lysed mouse tissues for WB analysis. We probed the WBs with rabbit monoclonal anti-NEDD8 from Millennium Pharmaceuticals (Mil10) or from Epitomics (Epitomics). Neither the Mil10 nor the Epitomics rabbit anti-NEDD8 monoclonal antibodies are believed to have nonspecific bands and both were able to detect primarily the same tissue specific banding patterns (Figure 4.4).

4.5.1.1 Heart

The main point of divergence between the two rabbit monoclonal antibodies against an N-terminal fragment of NEDD8 was observed in heart. There is a very pronounced anti-NEDD8 reactive band of about 40kD that appears stronger than the cullins when probed with Mil10. However, it is not recognized by Epitomics anti-NEDD8. These antibodies otherwise behave very similarly and we therefore concluded that this discrepancy was likely due to nonspecific interactions between Mil10 and a heartprotein. We decided to focus instead on a tissue such as testes with a high degree of neddylation but no such incongruity between the antibodies. We believed the putative substrates in such tissues were less likely to constitute nonspecific bands.

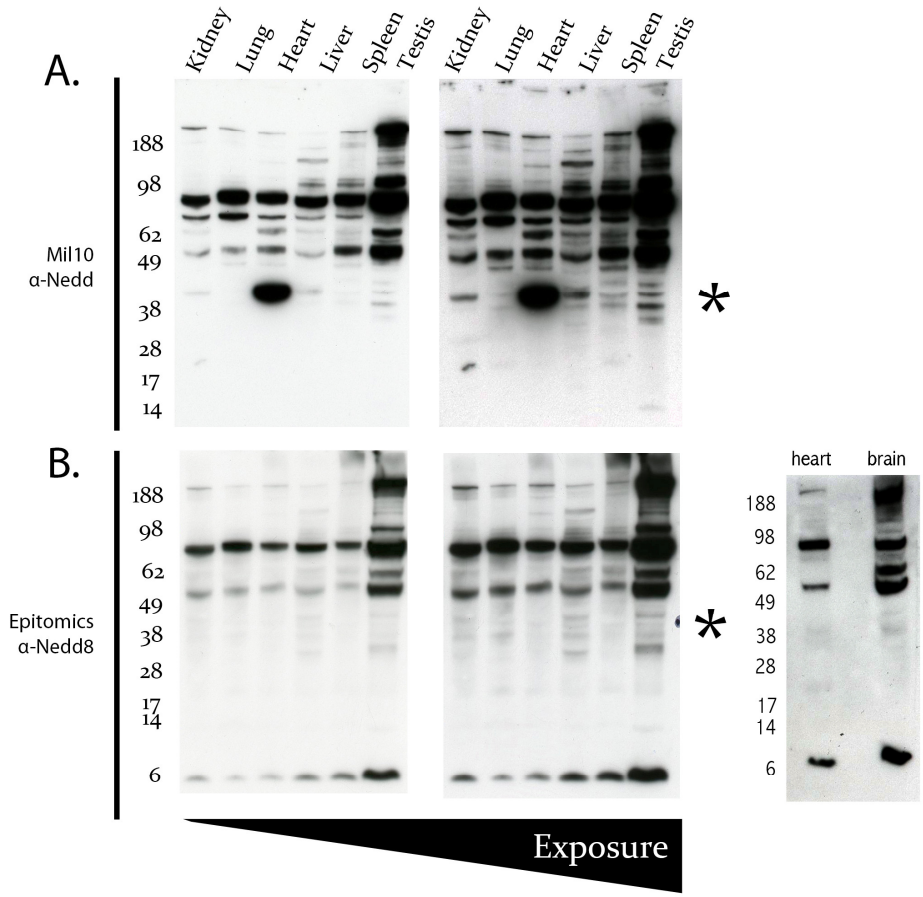


Figure 4.4. Mouse tissue panels blotted for NEDD8. 40 µg of protein from each tissuesuspended in reducing sample buffer separated by SDS-PAGE and blotted for NEDD8 using Mil10 (A) and Epitomics (B) anti-NEDD8 antibodies by WB. Possible nonspecific band noted with the * symbol.

4.5.1.2 Testes

Our main focus shifted to Testes. Not only did the UCHL3 knockout mouse have an abnormal heat shock response in testes (Kwon et al., 2004), but we also observed an elevated amount of anti-NEDD8 reactive species by WB using two different antibodies. Promisingly, there were some high molecular weight species (~250kD) too large to

correspond to the cullins or cullin degradation products. As these samples were all prepared under reducing conditions these bands cannot constitute cullin dimers.

4.5.1.3 Brain

There is also an increased banding pattern in brain. It is interesting to note that some of the anti-NEDD8 reactive bands with increased intensity in brain match up with some of the unidentified bands that were observed to be stable across various lysis buffers when cultured cells were checked for degradation products. These could represent genuine substrates. It is attractive to postulate that these neddylated proteins are associated in some way with the memory loss or the dorsal root ganglion degeneration phenotypes of the UCHL3 KO mouse. That hypothesis needs to be tested.

4.5.2 Substrate Identification

In order to ascertain whether we were observing genuine non-cullin substrates, it was necessary to develop a means of purifying proteins modified by NEDD8. We elected to pursue several affinity based purification techniques in parallel.

4.5.2.1 NEDD8 Affinity Matrix

First, we attempted to develop a NEDD8affinity matrix (NAM) by exploiting the specificity NEDP1 has for NEDD8 to selectively isolate neddylated proteins. This technique, discovered by Roland Hjerpe, involves using N-terminally taggedcatalytically dead NEDP1 (tagged-NEDP1 C163A) to pull on neddylated proteins. Dr. Hjerpe has demonstrated that the NAM was able to specifically isolate NEDD8 from a mixture of purified ubiquitin and NEDD8. I then took over the project and optimized this process for use on cell lysate. While the NAM can be used to purify neddylatedproteins, its greatest preference is for pulling down free NEDD8 (Figure 4.5). While the NAM may prove viable in the future when used in conjunction with

othertechniques, it is not a functional way to purify neddylated proteins from a complex mixture using current experimental design.

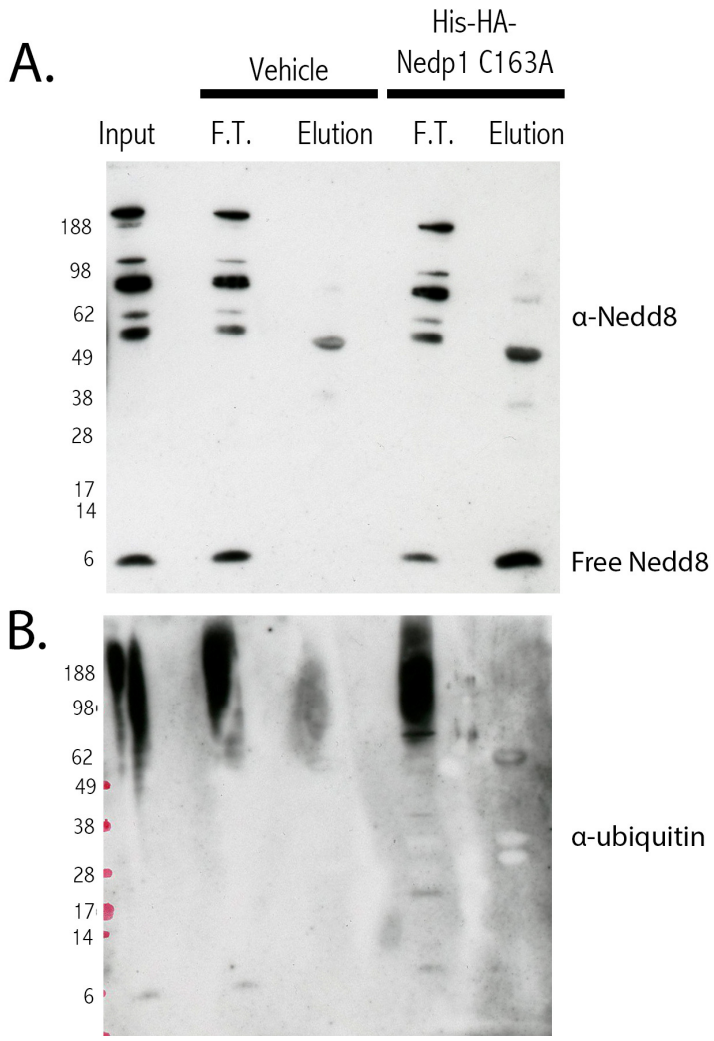


Figure 4.5. NAM used to purify neddylated proteins from HCT-116 cell lysate. Cells incubated in lysis buffer with His-HA-NEDP1 C163A or an equivalent volume of Ubiquigent buffer vehiclethen immunoprecipitated using HA-agarose beads. The pulldown was tested for the ability to isolate NEDD8 (A) and ubiquitin (B) associated proteins by WB. Although the NAM pulls down Nedd8 and some associated proteins, it does not pull down ubiquitin.

4.5.3 Immunoprecipitation

4.5.3.1 Antibody production

In parallel with the work developing the NAM pulldown procedure, we also worked to establish an IP procedure. In addition to the two commercial monoclonal anti-NEDD8 antibodies already discussed (Mil10 and Epitomics), two polyclonal anti-Nedd8 antibodies were specifically developed for this project.

4.5.3.2 Polyclonal anti-NEDD8

Polyclonal sheep and rabbit anti-NEDD8 antibodies were generated using the same antigen (residues 11-32 of human NEDD8) as with the monoclonals. These antibodies were tested by dot blot for specific NEDD8 interaction, ubiquitin cross-reaction, and generic interactions with large quantities of control protein (BSA). Both the sheep and the rabbit anti-NEDD8 are capable of specifically recognizing small quantities of native and denatured NEDD8 by dot blot (Figure 4.6 A). The rabbit anti Neddd8 may display some nonspecific interactions with high quantities of protein (~500 ng). To see if these results translated to WB the antibodies were tested at 1:500 overnight on membranes containing one lane with a sample where the NAE was functional and one lane with a sample where the NAE was inhibited by MLN4924. While the sheep polyclonal is able to recognize the cullins and to a limited extent, free NEDD8 in HCT116 lysate, it is much weaker than Mil10. The Rabbit anti-NEDD8 is stronger than the sheep and is perhaps comparable to Mil10 with the exception of 1 nonspecific band at ~40kD (Figure 4.6 B).

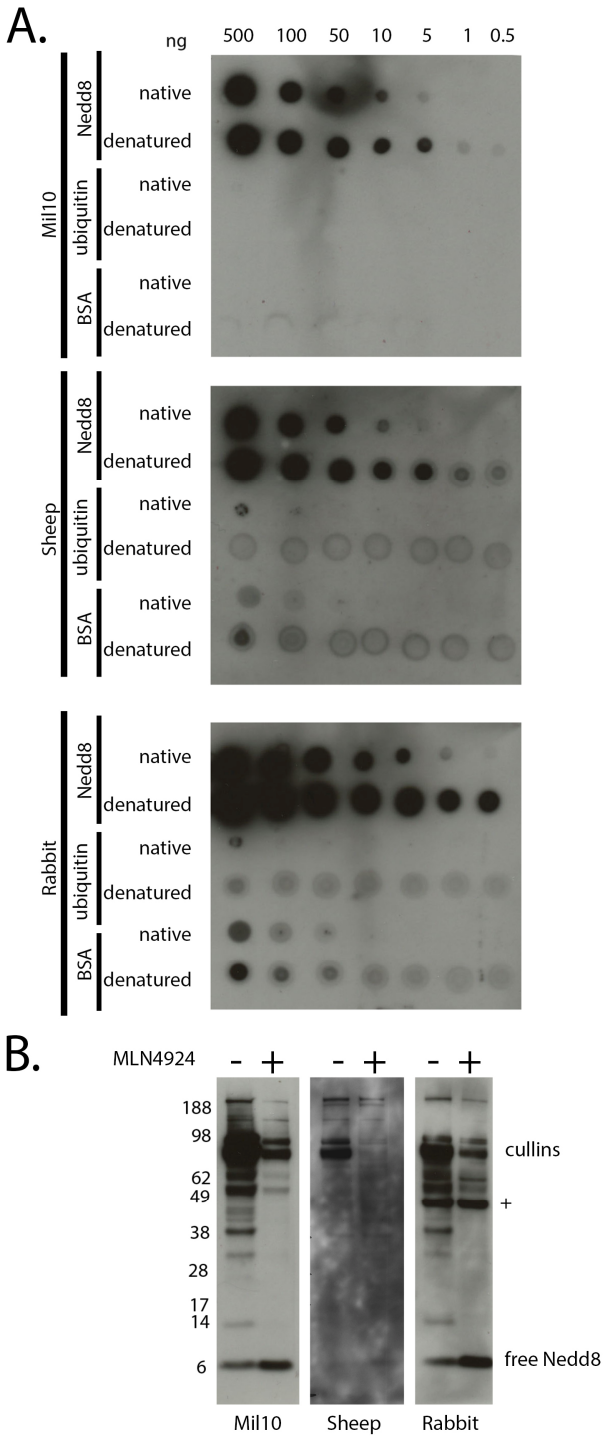


Figure 4.6. Polyclonal rabbit and sheep anti-NEDD8 characterization by dot blot and WB. The current standard, Mil10 was used for comparison. There is some nonspecific reaction to high levels of protein with the polyclonal antibodies (A). The rabbit polyclonal anti-Nedd8 is effective by WB compared to the Mil10 (B) however there is a nonspecific band (denoted by +).

4.5.3.3 Immunoprecipitation from testes: Non-denaturing

While methods of pre-enrichment like deneddylase knockdown may still be useful in attempting to identify neddylated proteins in cultured cells, we elected to IP from testes. Testes were chosen for two reasons. First it made our samples biologically relevant. Cancer cells can be wildly misregulated so use of endogenous tissue can help attain a clearer picture of what is happening in normal tissue. Secondly, the increased neddylation profile in testes WB meant that the putative non-cullin NEDD8 substrates are already enriched for in tissue samples.

For these experiments we extracted proteins from mouse testes and immunoprecipitated from those samples overnight using one of the anti-NEDD8 antibodies or preimmune IgG control. Under non-denaturing conditions, the Epitomics antibody was most efficacious and was therefore the primary antibody of choice. Even with 1mg of testes protein the Epitomics antibody was able to IP known NEDD8 pathway members including CUL9. Coverage was increased with increasing amount of protein in the IP. We have also tried 4mg, and 20mg IPs but the 1 and 4 mg IPs had the least background. Samples were separated by molecular weight using SDS-PAGE. Gels were either silver or Coomassie stained then cut into slices that were prepared for MS analysis.

4.5.3.3.1 MS Scores

Samples were run on an LTQ Orbitrap classic (Thermo). Hits were then analysed using MASCOT and peptides were filtered for an ion score higher than 28. Peptides for the common contaminants keratin, serum albumin, and immunoglobulin were excluded from analysis. A score above approximately 100 is considered a very solid hit. While scores can be compared within a single run they should not be compared quantitatively

between two different runs—for example COPB was assigned a score of 200 in the 1mg IP and 140 in the 4mg IP. From that we can extrapolate that COPB is a strong hit but not that the 1mg IP was better than the 4mg IP. In fact, for most proteins the scores would follow the opposite trend by scoring higher in the 4mg IP. That being said, knowing that there is a high degree of confidence for a hit is beneficial and some scores are included here to illustrate our relative confidence in the observed proteins.

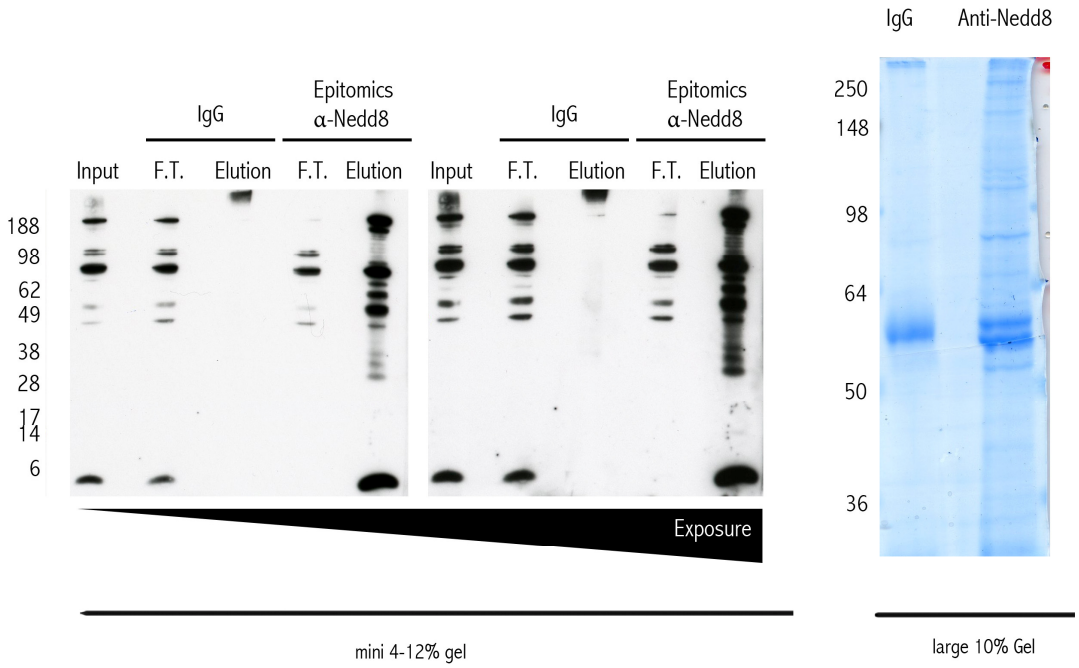


Figure 4.7. Epitomics anti-NEDD8 native IP. The WB and Coomassie stained gel obtained from immunoprecipitation of 4 mg of mouse testes proteins using 50 µg antibody overnight.

4.5.3.3.1.1 NEDD8 pathway members found in the non-denaturing IPs

Many expected pathway members were identified by MS of testes extract IPs including cullins (Table 4.1). First, we found NEDD8 peptides confirming that our technique was successful. We were also able to identify the NAE component ULA1. Surprisingly, the strongest hit amongst the cullins was CUL9. CUL9 is a very atypical cullin with no reported adaptor protein or substrate recognition protein (Sarikas et al., 2011). CUL9 has not been shown to make use of the typical RING domain proteins RBX1/2 all other cullins interact with (Sarikas et al., 2011). CUL9 plays a regulatory function for p53 and is known to dimerize with CUL7(Skaar et al., 2005). Of all the cullins, only CUL7 has not clearly been shown to be neddylated (Skaar et al., 2007). However CUL7 came down in these nondenaturing IPs. It should be noted that CUL7 consistently scored less than CUL9 and may be coming down as part of a heterodimer. Currently CUL7 is the only cullin not conclusively found to be neddylated (Sarikas et al., 2011). Other cullins including cullins 3, 4a, and 5 were identified in these native testes IPs as well.

NEDD8 Pathway
NEDD8
ULA1
Cullins 3, 4a, 5, 7, 9
COP9 signallosome
CAND1/2

Table 4.1.*NEDD8 pathway members identified in native NEDD8 IPs by MS. Data from three IPs was used to compile this list.*

4.5.3.3.1.1.1 CAND1

One surprising NEDD8 pathway component observed in these nondenaturing testes IPs was cullin-associated-NEDD8-dissociated 1 (CAND1). CAND1 has also been reported to associate with overexpressed GST-NEDD8(Jones et al., 2008). CAND1 binds to the cullin backbone of CRL complexes preventing both the binding of NEDD8 and substrate adaptors (Schmidt et al., 2009). As CAND1 is believed to exclude NEDD8

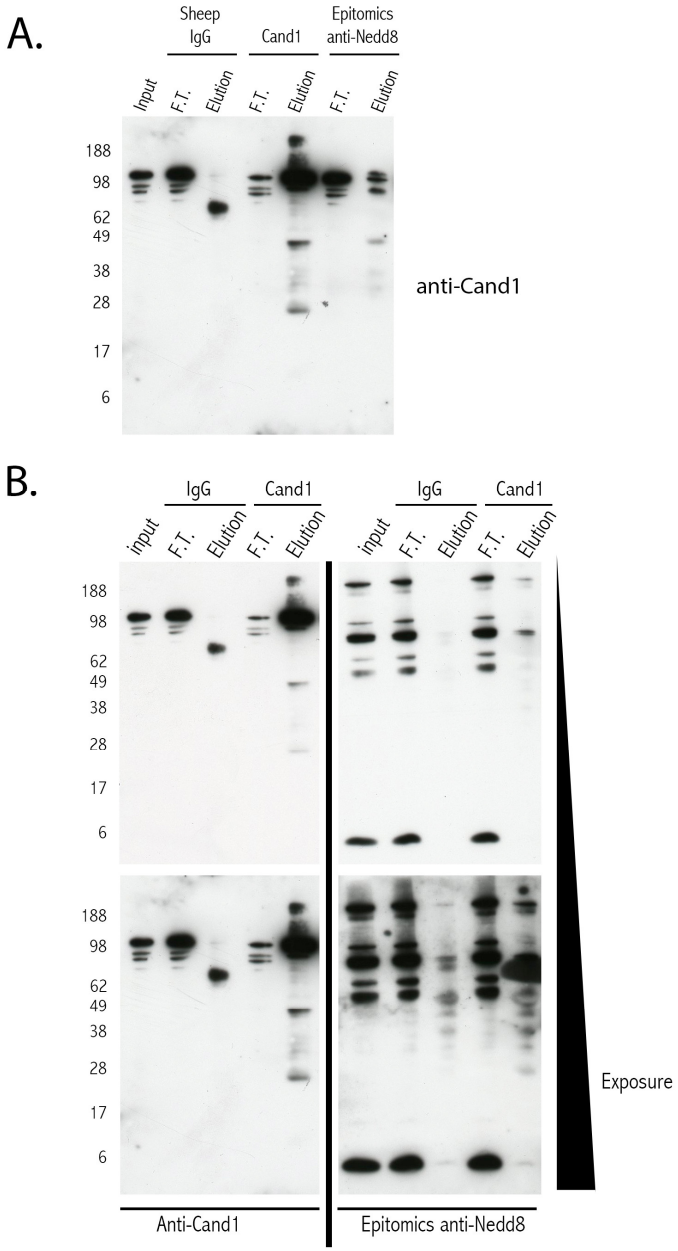


Figure 4.8. CAND1 reciprocal IPs. CAND1 was immunoprecipitated overnight with 200 uL of beads, 2mg of testes, 15ug antibody and a total volume of 750 uL. The standard wash and elution protocol was followed. The WBs done to confirm the efficacy of the IPs were then blotted with anti-CAND1 and Epitomics anti-NEDD8.The MS result that CAND1 comes down in native NEDD8 IPs is confirmed (A). There are NEDD8 reactive species that associate with CAND1 in non-denaturing IPs but these are not at the same molecular weight as CAND1 itself (B).

from the CRL complex we did not expect it to associate with NEDD8. Cullins have been known to dimerize, therefore it is possible that CAND1 is coming down associated with a cullin that is dimerized to a neddylated cullin.

In order to determine if CAND1 was itself neddylated or if it was merely associated with neddylated proteins we performed a reciprocal IP using anti-CAND1. Pulling on CAND1 results in a specific enrichment for some neddylated species. However these bands do not correspond to the molecular weight of CAND1 itself and are likely the proteins with which CAND1 complexes. Such behavior indicates that NEDD8 is not directly bound to CAND1 (Figure 4.8). We believe CAND1 binds to a cullin that is dimerized to a second neddylated cullin and is therefore associating with NEDD8 indirectly. This theory needs to be substantiated, perhaps through identification of the cullins involved.

4.5.3.3.1.2 Ubiquitin pathway members found in non-denaturing IPs

Ubiquitin associates with CRL complexes and has been reported, under conditions of overexpression, to form mixed chains with NEDD8(Leidecker et al., 2012). It is not surprising therefore, that ubiquitin comes down in this IP. What is surprising, is the association between NEDD8 and UBA1 the ubiquitin activating enzyme (UAE) and UBA1Y the mouse testes specific isoform of UBA1. The fact that these enzymes are identified strongly indicates that NEDD8 may be activated by the UAE at endogenous levels.

4.5.3.3.1.3 Other families of proteins in the NEDD8 associated proteome

Other families of proteins were also found in the non-denaturing testes IPs. There are numerous proteins involved in nuclear transport that were identified including exportins 1/2/7, importin 7/B1, and nucleophosmin.

Another recurring protein in both IPs is matrin-3 which is involved in retention of hyperedited RNA in the nucleus (DeCerbo and Carmichael, 2005). It forms a trimeric complex with NONO and SFPQ to perform this role (Salton et al., 2010; Zhang and Carmichael, 2001). Both of these other proteins also come down specifically. Proteins which have been linked to matrin-3 including importin 7, nucleophosmin, exportin1, DHX9, and HNRNPK (www.amigo.geneontology.org) are also found in these IPs (Table 4.2) lending credence to the solidity of this find. However none of these proteins has been found bearing a diglycine modification and it is unclear why these proteins associate with NEDD8. We are unsure if any of these proteins directly bind NEDD8.

	Epitomics		
protein	1 mg	4 mg	20 mg
antibody	50 ug	50 ug	200 ug
Matrin-3	700	892	709
FNONO		96	311
SFPQ		305	658
IPO7		56	230
NPM		860	721
XPO1	42	353	528

Table 4.2. *Matrin-3 associated proteins identified from non-crosslinked native NEDD8 IPs using the Epitomics anti-NEDD8 antibody. Settings for protein and antibody loading are used to distinguish the IPs. Scores for each protein in each IP are listed. All proteins were identified by more than one unique peptide*

4.5.3.3.1.3.1 Structural Maintainance of Chromosomes (SMC)

The SMC family of proteins has also been found to associate with NEDD8 (Table 4.3). The SMC proteins are, as their name implies, involved in regulation of chromosome maintenance. SMC2 and 4 are integral components of the condensin complex while SMC1A and SMC3 are components of the cohesin complex.

SMC1A is in all testes IPs. SMC2 and SMC3 are found strongly with the Epitomics antibody. SMC4, however, is not identified as robustly. Using the Mil10 and Epitomics antibodies, the signal for these SMC proteins is vastly decreased. Also noteworthy, is the observation that in a native mouse heart IP, SMC3 was observed. While only one peptide was seen, that peptide bore a diglycine modification on a lysine. It is possible that SMC3 is a substrate of neddylation but as we have not replicated this finding and the modification in the heart IP was based on a single peptide no conclusions can be drawn. One further caveat to any such identification is that if SMC3 were ubiquitylated, preparation of the peptides by trypsinization would leave the same diglycine modification as would be observed for neddylation.

Other members of condensin (NCAPD2, NCAPA, NCAPH, NCAPD3) and cohesin (RAD21, REC8, RAD21L, STAG2 and STAG3) (as listed by www.amigo.geneontology.org) were not found in the NEDD8 IPs. Confidence in our finding that NEDD8 and the SMC family are linked is strengthened by the work of Jones et al. demonstrating that the SMC family associated with overexpressed GST-NEDD8.We are unclear on why non-SMC complex components do not co-immunoprecipitate with the SMC proteins. We are also unsure why SMC proteins have been identified in the NEDD8 associated proteome. However given the repeatability

and published support for the interaction this may be a real association. Possible ways to improve this signal include using benzonase to help dissociate DNA bound proteins or nuclear enrichment before IP.

	Testes				Heart
	Epitomics			Rabbit	Mil10
protein	1 mg	4 mg	20 mg	20 mg	8 mg
antibody	50 µg	50 µg	200 µg	200 µg	20 µg
SMC1A	34	84	328	30	
SMC2		213	164		
SMC3		567	783	39	32
SMC4		53	97		

Table 4.3.*SMC proteins identified in non-denaturing NEDD8 IPs. IPs were done from mouse testes and mouse heart using the following anti-NEDD8 antibodies: Epitomics, rabbit polyclonal anti-NEDD8, and Mil10. IPs are further identified by amount of protein and antibody loaded. MASCOT scores are given as an indication of signal strength. Yellow boxes denote proteins identified by one unique peptide. Red box denotes a peptide identified by one peptide bearing a diglycine modification.*

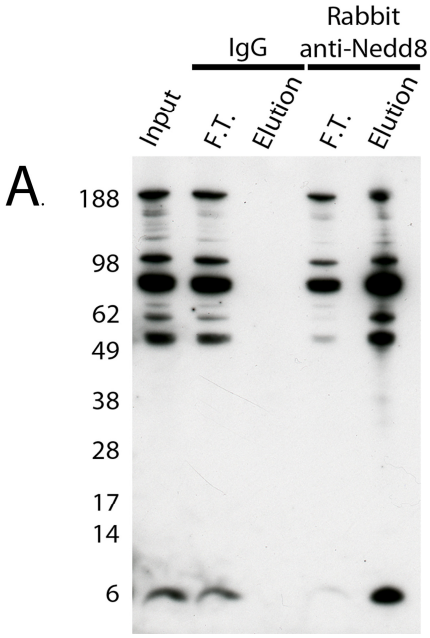
4.5.3.3.1.4 Coatomer

One of the highest scoring recurring proteins in the non-denaturing testes IPs was the coatomer family of proteins (Table 4.4). We observed components of the COPI complex which coat vesicles for retrograde transport from the Golgi to the Endoplasmic Reticulum (Lee and Goldberg, 2010). Specifically, coatomer γ2 (COPG2) was extremely high scoring in even in the IP of 1mg of testes extract as was the γ1 (COPG1) subunit (scoring 2388 and 1301 respectively). Other complex components also came down in multiple IPs including COPA, B, and B2. Another protein that came down in repeated IPs, N-terminal kinase like protein (NTKL), or mitosis-associated kinase-like protein NTKL, is known to bind COPB1 (Burman et al., 2008). It is therefore possible NTKL came down with the complex.

	Testes				Heart
	Epitomics			Rabbit	Mil10
protein	1 mg	4 mg	20 mg	20 mg	8 mg
antibody	50 ug	50 ug	200 ug	200 ug	20 ug
COPA	108	656	1083	502	
COPB	200	140	237		
COPB~	34	31	105		
COPE					
COPG1	1301	1943	1365		
COPG2	2388	3621	3276		
COPZ1					
COPZ2					
NTKL	718	1008	1273		
TMED3					
AP4E1					

Table 4.4. *Scores for coatomer and associated proteins that were found in non-denaturing NEDD8 IPs. The COP family are coatomer proteins. The bottom box, NTKL, TMED3, and AP4E1 are proteins reported to associate with the coatomer1 complex by The Gene Ontology AmiGO. MASCOT scores are given as an indication of signal strength. Yellow boxes denote proteins identified by only one unique peptide.*

The structure of the COP1 complex is remarkably similar to that of clathrin and its adaptors, which also serves to coat vesicles (Lee and Goldberg, 2010). Clathrin, and some of its adaptors, were also identified in these IPs albeit to a lesser extent. The clathrin adaptor subunit most like the γ subunit of coatomer, AP2 (Popoff et al., 2011), is known to selectively recruit ubiquitylated proteins (Kumar et al., 2007). It is possible that the enrichment for the COPγ subunit is due to a similar interaction with the ubiquitin pathway or direct ubiquitlyation of a COP1 subunit rather than direct interaction with NEDD8.



B.

Protein	Score
Cullin 3	229
Cullin 4A	255
Cullin 4B	298
Cullin 5	145
Cullin 9	962
Nedd8	683
Uba3	53
Ula1	53
Tdrd7	*5021*
Golga2	*636*

C.

Rank	Protein	Score
1	Tdrd7	5873
2	Cul9	5181
3	Golga2	4713

Figure 4.9. Denaturing NEDD8 IP. IP of 7.5 mg of mouse testes using 30 µg of rabbit polyclonal anti-NEDD8 is able to deplete free and conjugated NEDD8 as seen on WB (A). NEDD8 pathway members were identified by MS analysis. The two significant hits not associated with NEDD8 (red, B) were also found specifically with this polyclonal antibody in the top three hits of the native Rabbit-anti-NEDD8 IP (C).

4.5.3.4 Denaturing IPs

As NEDD8 modifies CRLs, we expect non-denaturing IPs like those described above to bring down NEDD8 or CRL associated proteins. In order to specifically narrow those findings to NEDD8 modifiers, denaturing IPS are required as they interfere with transient associations and enrich for covalently bound substrates. We again attempted to identify neddylated proteins using mouse testes extract. We lysed the testes in the same nondenaturing lysis buffer as with the nondenaturing IPs. We then TCA precipitated these proteins and resuspended them in denaturing lysis buffer. Denatured proteins were incubated with anti-NEDD8 antibody. The rabbit polyclonal anti-NEDD8 was much more effective at isolating denatured neddylated proteins than the

Epitomics antibody and was used for the denaturing IP that we then sent for MS analysis (Figure 4.9 A). We again elected to prepare our samples from a Coomassie stained gel. Samples were run on a LTQ Orbitrap Classic (Thermo) and spectra were analysed by MASCOT.

4.5.3.4.1 NEDD8 pathway members in the denaturing NEDD8 IP

As expected, we found NEDD8 pathway members and cullins in the denaturing NEDD8 IP (Figure 4.9 B). We identified NEDD8 in the molecular weight region for free NEDD8 and also in the region where the majority of cullins were identified. We were also able to identify both components of the NEDD8 E1 heterodimer (ULA1 and UBA3). We again were unable to identify the NEDD8 E2 UBC12. However we were able to strongly identify the main known substrates, the cullins. Cullins that were identified include cullins 3, 4A, 4B, and 5. Again we were able to identify CUL9 as the strongest hit among the cullins.

We could not identify cullins 1, 2, or 7. Cullins 1 and 2 were not identified in the nondenaturing IPs either. It is possible that these cullins are not expressed as highly as the others in testes or are not as highly neddylated as the other cullins in this tissue. The relative abundance and neddylation of cullins 1 and 2 will need to be checked directly to determine if this is the case. CUL7, however, was extremely high scoring in the nondenaturing IPs but is not present here. One possible reason for this disparity may be that as described by Skaar et. al, CUL7 is not actually neddylated. This experiment will need to be repeated and optimized to conclusively state that there is no neddylated CUL7 in testes.

4.5.3.4.2 Polyclonal rabbit anti-NEDD8 specific interactions

The strongest scoring hit in the rabbit anti-NEDD8 IPs both native and denatured was the Tudor domain containing protein 7 (TDRD7). The one native NEDD8 IP using the rabbit antibody that was sent for MS analysis, had very low coverage in general but TDRD7 scored extremely well (5873) (Figure 4.9 C). The top hit from the denaturing NEDD8 IP using the same antibody was also TDRD7 which scored more than 5 times higher than CUL9, the highest scoring cullin (5021 and 962 respectively). Due to the fact that TDRD7 appears stronger than the known substrates of NEDD8 and is only identified with one of the two antibodies used for IP, we believe that TDRD7 binds nonspecifically to the Rabbit anti-NEDD8 antibody. This theory has not been directly tested.

There are not many other non-cullin hits from the denaturing IP. In addition to TDRD7, we have also identified with confidence one other protein: Golgin subfamily A, Member 2 (GOLGA2). When we BLAST the peptide used to generate the Rabbit anti-NEDD8 antibody, GOLGA2 is found. This leads us to believe that this protein is coming down in IPs as a result of nonspecific binding of the antibody rather than because of a genuine NEDD8 interaction. Like what we observe for TDRD7, the non-denaturing NEDD8 IP further supports this analysis as GOLGA2 is the third highest scoring hit, after TDRD7 and CUL9 (Figure 4.9 C), but is not similarly immunoprecipitated by the Epitomics antibody.

We have not identified any strong contenders for non-cullin substrates using this denaturing IP. However, coverage is low because we have not identified all of the cullins, Ubc12, or probable NEDD8 substrates like p73 or BCA3. It is possible that

there are no non-cullin substrates. It is also possible that such substrates exist but are beyond the detection limit of the protocol used for this IP. This experiment will need to be optimized in order to conclude one way or the other.

Chapter 5: Discussion

5.1 Yeast Rub1 turnover

As reiterated in the introduction of recent work done byBuchsbaum et al., 2012, on FAT10 degradation, the common belief in NEDD8 field is that NEDD8 is rapidly turned over. We report here that tagging, overexpression, and mutation of the diglycine motif induce rapid UPR mediated Rub1 turnover in yeast. However, using a direct antibody against Rub1 we have been able to demonstrate that this observation is an artifactas both tagging and mutation destabilize the protein. Endogenous Rub1 is actually quite stable.

Possible implications of this finding might generalize to ubiquitin and other UBLS. Studies where the diglycine motif has been mutated might have erroneously made the same assumptions about stability as we have. It is possible that the diglycine mutations and deletions have resulted in observed half-lives that are quicker than endogenous turnover rates. One possible explanation for this destabilization comes from NMR studies of ubiquitin stability. Kitahara et al., 2006a, reports that at high temperatures the short unstructured region bearing the diglycine motif is one of the first to become disordered. While we are not inducing heat stress here, this mutation could encourage instability by mimicking that condition.

Another contributing factor to Rub1 instability was the use of a tag. Even the short HA tag could be cleaved from the N-terminus of Rub1. The tag might also lead to unfolding and degradation via the UPR. Using a tagged system was a flawed approach both

because of this cleavage and because of destabilization of the protein. One possible explanation for this destabilization is that the N-terminal region is the first to fold in ubiquitin, and likely also in Rub1. It is upon this initial fold that all the others resolve(Mishra et al., 2009; Zerella et al., 1999). Rub1 is very similar to ubiquitin structurally but has decreased structural integrity when compared ubiquitin (Kitahara et al., 2006b). Interfering with the endogenous N-terminal fold by tagging may further destabilize the folding of Rub1. It is possible that many other proteins have been mistakenly attributed to the UPR because of tag induced unfolding. Based on the results observed with Rub1, we would recommend that tagging should only be used in cases where it is unavoidable and should be avoided altogether in studies of protein turnover.

In order to truly observe Rub1 turnover a radioactive pulse chase assay may be required. This technique will avoid the pitfalls of tagging and mutation already described. If a cycloheximide chase is not used in conjunction with this technique, there should be even less concern that an artifact is being analyzed. If the turnover of Rub1 is studied in the future, this is one technique that we would recommend.

With respect to endogenous Rub1, we were able to make a few novel observations. First, deletion of the Rub1 pathway enzymes *uba3* and *ubc12* results in an accumulation of free Rub1 on WB. In these strains, Rub1 cannot become conjugated to substrates. As Rub1 is not turned over quickly, we believe the Rub1 that would normally bind to substrates is simply allowed to accumulate in the free form. If Rub1 levels were being tightly regulated we would not expect this accumulation. We believe the fact that Rub1

appreciably increases in such strains indicates that Rub1 levels are not under strict control to remain at a WT levels.

A second interesting observation is that Rub1 is stable over the course of a 7hour cycloheximide chase. Therefore, Rub1 does not have the short half-life we initially believed it did. This finding is further substantiated in overexpression work where the tag free construct is stable when compared to its tagged counterpart. Our initial hypothesis that regulation of speedy Rub1 turnover provides a means of preventing cross talk with ubiquitin enzymes is therefore incorrect. Furthermore, the current belief in the field, that NEDD8 is turned over quickly, does not hold true in yeast.

It would be interesting to see if the destabilizations observed with mutations and tagging of Rub1 similarly destabilized other UBLs when compared to their wild type counterparts. One possible way of testing this is by using a pulse chase system with heavy amino acids followed by immunoprecipitation of yeast collected at various time points. Samples could then be analyzed by MS in order to determine quantitatively how much of the heavy Rub1/NEDD8 remains free or associated with substrates at any given time point. To determine if conjugated Rub1/NEDD8 has a different half-life than free Rub1/NEDD8, the free Nedd8 could be separated using a low molecular weight spin filter. The pool of free and conjugated NEDD8 would then be separate and degradation rates of the labeled Rub1/Nedd8 could be compared over time.

While we were unable to ascertain the pathway by which Rub1 was turned over, we were able to show that variations in the pool of free Rub1 are not corrected immediately.

Rub1 levels do not appear to be under extremely tight control. One possible explanation for this observation is that a small level of crosstalk with the UAE is natural. In conditions of stress, such as heat shock (Hanna et al., 2003), ubiquitin pools are depleted. In order to temporarily alleviate stress on the ubiquitin system it might be advantageous for Rub1 to become conjugated via the ubiquitin pathway. In emergencies Rub1 might therefore be temporarily utilized to assist to the beleaguered ubiquitin system so that the cells can survive until the stress is removed. It is possible that, as we have suggested (Hjerpe et al., 2012a), relative amounts of free ubiquitin serve as the factor responsible for minimizing Rub1/ubiquitin crosstalk. This would ensure that Rub1 could serve as an emergency back-up system for use by the ubiquitin pathway enzymes when and as needed.

5.2 Yeast Rub1 Substrates

Although non-cullin substrates of Rub1 (including Lag2(Siergiejuk et al., 2009)) have been reported, we were unable to identify any such substrates at the endogenous level in our IP experiment. The only proteins confidently identified co-immunoprecipitating with Rub1, were CRL complex components. Cullins are the major accepted substrate of neddylation (Rabut and Peter, 2008) and we were expecting them to be found. Similarly the identification of F-box components lends credence to our analysis as they form part of cullin-RING-ligases. We are unable to say if non-cullin substrates exist at an endogenous level. If such substrates exist, we were unable to identify them. One possible reason for this is that coverage was not high enough and these alternative substrates are low abundant. Another possible explanation is that there are no non-cullin substrates in yeast under endogenous unstressed conditions.

Possible ways to look for novel roles of Rub1 in yeast include inducing stresses such as DNA damage and heat shock and then looking for Rub1 conjugates. Such experiments would require a control strain lacking Rub1 conjugation enzymes such as Uba3 or Ubc12. This way, possible conjugates could be specifically attributed to the ubiquitylation or the neddylation machinery. One other possible additional control strain is the TS UAE strain. The limitation of that control is that by impairing the whole ubiquitylation pathway, more off target effects may be induced than those that might be observed in the *uba3* and *ubc12* deletion strains. A series of similar experiments in mammalian cells might be simpler as MLN4924 can inhibit the NAE specifically. Unfortunately this drug is not useful on yeast (most likely because it cannot pass through the cell wall).

5.3 Mammailan NEDD8

In cultured cells we are able to observe multiple neddylated species. Many of these fall below the molecular weights of the cullins and might therefore be degradation products. Lysis in different lysis buffers helped us identify anti-NEDD8 reactive bands on WB that might represent degradation products. There are a couple bands between 49 and 62 kD which were of particular interest because they seem unaffected by changes in lysis buffer. This could mean that these proteins are not being cleaved after cell lysis. It is still possible that they are cullin degradation products formed in vivo. This analysis is supported by the CSN5 siRNA experiment. CSN5 deneddylates cullins. Therefore when CSN5 is knocked down, neddylated cullins are enriched. We observed that the bands of interest are also enriched in this experiment. It is possible therefore that

cullins are cleaved in vivo and degraded as fragments. It is also possible that these substrates are independent of the cullins and also deneddylated by CSN5. Knocking down the deneddylase NEDP1 increases the neddylation of these bands as well. Therefore these bands could constitute novel substrates or NEDP1 could be deneddylating cullin fragments. The proteins represented by these bands could also play a tissue specific role as they are strongly represented in brain tissue.

The attempt to purify these and other neddylated proteins using the NAM was initially promising. It could specifically recognize NEDD8 over ubiquitin in vitro and purify free NEDD8 and lower molecular weight NEDD8 conjugates. I have concluded that the current NAM probes are not a viable route for purification of neddylated proteins. Despite repeated attempts using different settings and tags we did not efficiently purify the lower abundant NEDD8substrates we were after. The TUBES system which was the basis for the NAM theory relies upon tandem repeats of ubiquitin associated domains to increase ubiquitin binding. Within the Kurz lab it has been suggested that is possible that using tandem repeats of catalytically dead NEDP1 could similarly result in increased NEDD8 binding. However, in my opinion, this is unlikely to parallel the success with ubiquitin as NEDD8 is not as frequently bound in chains as ubiquitin is. I have postulated thatmixing ubiquitin binding domains with NEDP1 C163A could help pull down mixed NEDD8 ubiquitin chains. There are possible problems with this approach as well because the size of NEDP1 could separate NEDD8 binding from ubiquitin binding. The other possible use for the NAM is in conjunction with another purification technique. For example, it could be used following immunoprecipitation to

reduce IgG in the elution. However as it stands, I believe this approach should be abandoned.

The main challenge to specifically identifying the neddylated bands by IP arises because the MW of the proteins is almost the same as that of the IgG heavy chain. On silver or Coomassie stained gels, it is difficult to distinguish specific bands from the heavy chain of the IgG in this MW region. MS analysis does not solidly identify these proteins from bands cut from these regions either. One way of combatting this problem is by crosslinking the antibody to beads. Once crosslinking is successful, dependence on gels will be eliminated and the contribution of IgG to overall protein eluted will be minimized. Although attempts were repeatedly made using various crosslinkers, we have had difficulty crosslinking the rabbit anti-NEDD8 antibody to beads for this purpose. This process can be optimized. The Epitomics antibody had more luck with respect to crosslinking but it was not useful for denaturing IPs. Therefore future work should focus on crosslinking the polyclonal rabbit anti-NEDD8 so that these bands of interest can be identified.

Another way to purify these proteins is to first enrich for them. A recently generated NEDP1 knock-in cell line may do just that. Lysate from this strain may be immunoprecipitated to purify neddylated proteins. Stable isotope labeling by amino acids in cell culture (SILAC) analysis may also be used to specifically label and compare the proteins that are differentially regulated in this cell line when compared to a cell line with WT NEDP1. In the SILAC approach, one cell line is grown in heavy media while one is grown in light. For example, the NEDP1 knock-in cell line is grown

in light media and the WT cell-line in heavy media. The same amount of protein from each condition is combined and processed together. This joint sample can then be analyzed by MS and the peptides bearing heavy amino acids can be quantitatively compared to the peptides bearing light amino acids. In this way, the two cell lines can be directly compared to one another in an unbiased manner. In order to eliminate possible media based artifacts, the experiment is repeated with the inverse cell line media assignment (in this case NEDP1 knock in grown in heavy media and WT grown in light). The proteins that are upregulated or downregulated by NEDP1 knock in should remain consistent regardless of the media they are grown in. If NEDD8 is signaling for degradation or stabilization of any substrates, SILAC analysis will be able to demonstrate that.

Another important line of investigation with the knock in cell line would be to induce certain cell stresses and to observe if new conjugates are formed. In overexpression experiments, for example, DNA damage has been associated with NEDP1(Watson et al., 2010). As NEDD8 is the only known substrate of this protease it is possible that by inducing damage and then checking for novel neddylated proteins something new may be discovered.

Another telling experiment that may be done with this cell line, possibly in conjunction with one of the other approaches, is to isolate different cellular components. BCA3, for example, has only been reported to be neddylated in the nucleus and is also a reported substrate of NEDP1(Gao et al., 2006). Therefore, enriching for a nuclear fraction might help confirm this finding endogenously. It also might help identify other neddylated

nuclear proteins. Inclusion of a buffer component such as benzonase could help isolate DNA associated proteins which may be neddylated. During most of the cell cycle, NEDD8 is primarily localized to the nucleus (Kamitani et al., 1997; Kurz et al., 2002)so it is possible that there are more non-cullin substrates that may be found specifically in that organelle. Another membrane bound organelle of interest is the Golgi bodies. Golgi related proteins such as the coatomer proteins are enriched in our non-denaturing IPs. It is possible that there are some associated substrates that will be clearer following the knockdown of the NEDP1 deneddylase. This cell line may be the key to properly enriching for these putative non-cullin substrates.

With respect to CAND1, there are some obvious experiments that should be undertaken in the future. First, the cullins we believe are linking CAND1 to NEDD8 in NEDD8 IPs should be identified. Some possible ways to address this problem are by WB, MS, or a combination of the two. One possible way of doing this is to do two sequential IPs: first, IP for CAND1 under native conditions, and then using the eluent, IP for NEDD8. The cullins that associate with CAND1 but not with NEDD8 are likely bound to CAND1. The cullins that come down in the CAND1 IP and in the subsequent NEDD8 IP are likely to represent the neddylated cullin. By comparing the cullins found in bothIPs, it is possible to find out which cullin dimer pair is linking CAND1 to NEDD8. These findings can then be confirmed by reciprocal IPs of the cullins or MS analysis of the CAND1 IP. Once the composition of the complex is known, the function of the CAND1 bound cullin/NEDD8 bound cullin complex could then be investigated. At the moment it is unclear what CAND1 is doing in this complex. One possibility is that the CAND1 bound cullin somehow serves to increase or decrease the ubiquitylation activity

of the neddylated cullin. Whether it actually plays such a role in vivo will need to be tested.

One other interesting observation from the NEDD8 IPS was the interaction of the UAE with endogenous NEDD8. This interaction enhances our belief that there is a low level of endogenous neddylation occurring in a UAE mediated pathway. Based on the fact that the UAE may be charged withNEDD8 in vivo, it is possible that substrates identified by overexpression of NEDD8 might be genuinely neddylated via the UAE under endogenous conditions. This observation also further supports the theory that NEDD8 is being maintained as a free pool against conditions of stress where it may provide support for a strained ubiquitin system. However this theory will need to be tested by using various cell stresses without NEDD8 overexpression. Further work could include comparing UAE and NAE charging under various conditions. The separation between the ubiquitylation and neddylation pathways may not be as distinct as previously believed.

The observation that UCHL3 knockdown resulted in neddylation and cell cycle defects was interesting for many reasons. First, tissues bearing defects in the KO mouse such as brain (Wood et al., 2005) and testes (Kwon et al., 2004) have abnormally high neddylation profiles when compared to other tissues like lung and kidney. We believe that in the UCHL3 KO mouse these proteins are either no longer neddylated or minimally neddylated and that results in the observed phenotypes. This theory will need to be tested by directly examining the UCHL3 KO mice. Towards this end, it would be of interest to examine the neddylation profile in tissues of this UCHL3 KO

mouse compared to WT littermates. The ubiquitylation profile has already been examined in this manner and is ostensibly the same between KO and WT (Setsuie et al., 2009a). We believe that the phenotypes likely result from neddylation specific problems. If this can be proven it would demonstrate that NEDD8 could be responsible for the observed phenotypes.

Another possible outcome of such investigations would be to confirm the second half of the UCHL3 story. This is the connection between UCHL3 and sickness. While this protein is strongly linked to cancers (Miyoshi et al., 2006; Nam et al., 2003), there is also a reported connection with hypoxia and AD (Dennissen et al., 2011). It was this link in conjunction with the tissue specific phenotypes of the UCHL3 KO mouse that really kept the tissue specific idea in the forefront of this work despite the fact that the focus of this laboratory is not related to AD, hypoxia, or UCHL3 and our model systems do not typically include mice or tissues in general. There are some promising similarities between AD and UCHL3 impairment such as short-term memory loss, atypical gait, UBB+1 accumulation and ataxia. The idea behind this connection is in its infancy as these similarities do not prove causation. Much more work would be needed to illuminate any possible interactions.

There are other experiments that can be tested in the immediate future. For example, the finding that there are tissue specific neddylation profiles by WB means that there could be tissue specific neddylated and NEDD8 associated proteomes. These differences have never, to my knowledge, been examined in a tissue specific manner at

the endogenous level. It could be important to find out if there are tissue specific means of regulating NEDD8 conjugation or CRL substrate selection.

Admittedly, we have not been able to identify non-cullin substrates of neddylation. This failure may be due to incomplete coverage of the neddylated and NEDD8 associated proteomes. Alternatively there may be no neddylatd non-cullin proteins under endogenous conditions. Coverage of the denaturing NEDD8 IP must be increased. Then this optimized method may be used on various different tissue types. Other possible routes that may be taken to identify novel substrates include the use of different stress conditions. Leidecker et al., 2012, demonstrated that there may be UAE dependent increases in NEDD8 conjugation following stress situations. However, there has not been a comprehensive study of which proteins are most effected and if any stress induced conjugates are NAE dependent. The last means of enriching neddylated proteins that will be investigated in the immediate future is elimination of deneddylase activity. The NEDP1 knock-in cell line will prove invaluable in future work. It will have to be characterized and examined for neddylated proteins. While there are no guarantees that this future work will identify novel substrates of neddylation, the techniques are in place to continue the search.

Chapter 6: References

(2009). pYES2/CT, pYES3/CT, and pYC2/CT: Yeast expression vectors with C-terminal tags and auxotrophic selection markers, Invitrogen, ed. (Carlsbad, CA, USA).

Abcam. Stripping for Reprobing. In Protocols and Troubleshooting Tips (Cambridge, UK, Abcam).

Abida, W.M., Nikolaev, A., Zhao, W., Zhang, W., and Gu, W. (2007). FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity. The Journal of biological chemistry *282*, 1797-1804.

Armstrong, J. (2010). Yeast vacuoles: more than a model lysosome. Trends in cell biology *20*, 580-585.

Bennett, E.J., Rush, J., Gygi, S.P., and Harper, J.W. (2010). Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. Cell *143*, 951-965.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry *72*, 248-254.

Broemer, M., Tenev, T., Rigbolt, K.T., Hempel, S., Blagoev, B., Silke, J., Ditzel, M., and Meier, P. (2010). Systematic in vivo RNAi analysis identifies IAPs as NEDD8-E3 ligases. Molecular cell *40*, 810-822.

Buchsbaum, S., Bercovich, B., and Ciechanover, A. (2012). FAT10 is a proteasomal degradation signal that is itself regulated by ubiquitination. Molecular biology of the cell *23*, 225-232.

Burman, J.L., Bourbonniere, L., Philie, J., Stroh, T., Dejgaard, S.Y., Presley, J.F., and McPherson, P.S. (2008). Scyl1, mutated in a recessive form of spinocerebellar neurodegeneration, regulates COPI-mediated retrograde traffic. The Journal of biological chemistry *283*, 22774-22786.

Carlson, N., and Rechsteiner, M. (1987). Microinjection of ubiquitin: intracellular distribution and metabolism in HeLa cells maintained under normal physiological conditions. The Journal of cell biology *104*, 537-546.

Carlson, N., Rogers, S., and Rechsteiner, M. (1987). Microinjection of ubiquitin: changes in protein degradation in HeLa cells subjected to heat-shock. The Journal of cell biology *104*, 547-555.

Chauhan, D., Hideshima, T., and Anderson, K.C. (2005). Proteasome inhibition in multiple myeloma: therapeutic implication. Annual review of pharmacology and toxicology *45*, 465-476.

Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993). Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MAT alpha 2 repressor. Cell *74*, 357-369.

Chua YS, Boh BK, Ponyeam W, Hagen T (2011) Regulation of Cullin RING E3 Ubiquitin Ligases by CAND1 In Vivo. PLoS ONE 6(1): e16071.

Ciechanover, A. (2005). Proteolysis: from the lysosome to ubiquitin and the proteasome. Nature reviews Molecular cell biology *6*, 79-87.

Chin DT, Kuehl L, Rechsteiner M. Conjugation of ubiquitin to denatured hemoglobin is proportional to the rate of hemoglobin degradation in HeLa cells. Proc Natl Acad Sci U S A 1982; 79:5857-61

Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V., and Deshaies, R.J. (2002). Role of predicted metalloprotease motif of Jab1/CSN5 in cleavage of NEDD8 from Cul1. Science *298*, 608-611.

David, Y., Ziv, T., Admon, A., and Navon, A. (2010). The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. The Journal of biological chemistry *285*, 8595-8604.

DeCerbo, J., and Carmichael, G.G. (2005). Retention and repression: fates of hyperedited RNAs in the nucleus. Current opinion in cell biology *17*, 302-308.

Dennissen, F.J., Kholod, N., Hermes, D.J., Kemmerling, N., Steinbusch, H.W., Dantuma, N.P., and van Leeuwen, F.W. (2011). Mutant ubiquitin (UBB+1) associated with neurodegenerative disorders is hydrolyzed by ubiquitin C-terminal hydrolase L3 (UCH-L3). FEBS letters *585*, 2568-2574.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annual review of biochemistry *78*, 399-434.

Duda, D.M., Borg, L.A., Scott, D.C., Hunt, H.W., Hammel, M., Schulman, B.A. (2008). Structural Insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation. Cell 6, 995-1006.

Edelmann, M.J., Nicholson, B., and Kessler, B.M. (2011). Pharmacological targets in the ubiquitin system offer new ways of treating cancer, neurodegenerative disorders and infectious diseases. Expert reviews in molecular medicine *13*, e35.

Eisele, F., and Wolf, D.H. (2008). Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. FEBS letters *582*, 4143-4146.

Ferro, A., Carvalho, A.L., Teixeira-Castro, A., Almeida, C., Tome, R.J., Cortes, L., Rodrigues, A.J., Logarinho, E., Sequeiros, J., Macedo-Ribeiro, S.*, et al.* (2007). NEDD8: a new ataxin-3 interactor. Biochimica et biophysica acta *1773*, 1619-1627.

Finley, D., Ulrich, H.D., Sommer, T., and Kaiser, P. (2012). The ubiquitin-proteasome system of Saccharomyces cerevisiae. Genetics *192*, 319-360.

Frickel, E.M., Quesada, V., Muething, L., Gubbels, M.J., Spooner, E., Ploegh, H., and Artavanis-Tsakonas, K. (2007). Apicomplexan UCHL3 retains dual specificity for ubiquitin and NEDD8 throughout evolution. Cellular microbiology *9*, 1601-1610.

Fushman, D., Wilkinson, K.D. (2011). Structure and recognition of polyubiquitin chains of different lengths and linkage. F1000 Bio Reports.

Gan-Erdene, T., Nagamalleswari, K., Yin, L., Wu, K., Pan, Z.Q., and Wilkinson, K.D. (2003). Identification and characterization of DEN1, a deneddylase of the ULP family. The Journal of biological chemistry *278*, 28892-28900.

Gao, F., Cheng, J., Shi, T., and Yeh, E.T. (2006). Neddylation of a breast cancer-associated protein recruits a class III histone deacetylase that represses NFkappaB-dependent transcription. Nature cell biology *8*, 1171-1177.

Gardner, R.G., Nelson, Z.W., and Gottschling, D.E. (2005). Degradation-mediated protein quality control in the nucleus. Cell *120*, 803-815.

Geng, F., and Tansey, W.P. (2008). Polyubiquitylation of histone H2B. Molecular biology of the cell *19*, 3616-3624.

Goldenberg, S.J., Cascio, T.C., Shumway, S.D., Garbutt, K.C., Liu, J., Xiong, Y., and Zheng, N. (2004). Structure of the CAND1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. Cell *119*, 517-528.

Gong, L., Yeh, E.T.H. (1999) Identification of the activation and conjugation enzymes of the NEDD8 conjugation pathway. JBC 274, 12036-12042.

Gong, L., Kamitani, T., Millas, S., and Yeh, E.T. (2000). Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins. The Journal of biological chemistry *275*, 14212-14216.

Hanna, J., Leggett, D.S., and Finley, D. (2003). Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. Molecular and cellular biology *23*, 9251-9261.

Hemelaar, J., Borodovsky, A., Kessler, B.M., Reverter, D., Cook, J., Kolli, N., Gan-Erdene, T., Wilkinson, K.D., Gill, G., Lima, C.D.*, et al.* (2004). Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. Molecular and cellular biology *24*, 84-95.

Hicke, L. (1997). Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. FASEB 11, 1215-1226.

Hipp, M.S., Raasi, S., Groettrup, M., and Schmidtke, G. (2004). NEDD8 ultimate buster-1L interacts with the ubiquitin-like protein FAT10 and accelerates its degradation. The Journal of biological chemistry *279*, 16503-16510.

Hjerpe, R., Aillet, F., Lopitz-Otsoa, F., Lang, V., England, P., and Rodriguez, M.S. (2009). Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. EMBO reports *10*, 1250-1258.

Hjerpe, R., Thomas, Y., Chen, J., Zemla, A., Curran, S., Shpiro, N., Dick, L.R., and Kurz, T. Changes in the ratio of free NEDD8 to ubiquitin triggers NEDDylation by ubiquitin enzymes. Biochem J *441*, 927-936.

Hjerpe, R., Thomas, Y., Chen, J., Zemla, A., Curran, S., Shpiro, N., Dick, L.R., and Kurz, T. (2012a). Changes in the ratio of free NEDD8 to ubiquitin triggers NEDDylation by ubiquitin enzymes. The Biochemical journal *441*, 927-936.

Hjerpe, R., Thomas, Y., and Kurz, T. (2012b). NEDD8 overexpression results in neddylation of ubiquitin substrates by the ubiquitin pathway. Journal of molecular biology *421*, 27-29.

Hodgins, R.R., Ellison, K.S., and Ellison, M.J. (1992). Expression of a ubiquitin derivative that conjugates to protein irreversibly produces phenotypes consistent with a ubiquitin deficiency. The Journal of biological chemistry *267*, 8807-8812.

Hopper, L.M.M.a.J.E. Inducible Expression Cassettes in Yeast: GAL4, Vol 62 (Humana Press).

Howlett (2003). Protein Misfolding in Disease: Cause or Response? Curr Med Chem--Immun, Endoc & Metab Agents, 371-383.

Huang, D.T., Ayrault, O., Hunt, H.W., Taherbhoy, A.M., Duda, D.M., Scot, D.C., Borg, L.A., Neale, G., Murray, P.J., Roussel, M.F., Schulman, B.A. (2009). E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification. Mol Cell 33(4), 483-495.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of Escherichia coli with plasmids. Gene *96*, 23-28.

Jackson, S.E. (2006). Ubiquitin: a small protein folding paradigm. Organic & biomolecular chemistry *4*, 1845-1853.

Jentsch, S., and Pyrowolakis, G. (2000). Ubiquitin and its kin: how close are the family ties? Trends in cell biology *10*, 335-342.

Jones, J., Wu, K., Yang, Y., Guerrero, C., Nillegoda, N., Pan, Z.Q., and Huang, L. (2008). A targeted proteomic analysis of the ubiquitin-like modifier NEDD8 and associated proteins. Journal of proteome research *7*, 1274-1287.

Kamitani, T., Kito, K., Nguyen, H.P., and Yeh, E.T. (1997). Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. The Journal of biological chemistry *272*, 28557-28562.

Kitahara, R., Okuno, A., Kato, M., Taniguchi, Y., Yokoyama, S., and Akasaka, K. (2006a). Cold denaturation of ubiquitin at high pressure. Magnetic resonance in chemistry : MRC *44 Spec No*, S108-113.

Kitahara, R., Yamaguchi, Y., Sakata, E., Kasuya, T., Tanaka, K., Kato, K., Yokoyama, S., and Akasaka, K. (2006b). Evolutionally conserved intermediates between ubiquitin and NEDD8. J Mol Biol *363*, 395-404.

Kragt, A., Voorn-Brouwer, T., van den Berg, M., and Distel, B. (2005). The Saccharomyces cerevisiae peroxisomal import receptor Pex5p is monoubiquitinated in wild type cells. The Journal of biological chemistry *280*, 7867-7874.

Kumar, S., Tomooka, Y., Noda, M. (1992). Identification of a set of genes with developmentally down-regulated expression in the mouse brain. BBRC 3, 1155-1161.

Kumar, B., Lecompte, K.G., Klein, J.M., and Haas, A.L. (2010). Ser(120) of Ubc2/Rad6 regulates ubiquitin-dependent N-end rule targeting by E3{alpha}/Ubr1. The Journal of biological chemistry *285*, 41300-41309.

Kumar, K.G., Barriere, H., Carbone, C.J., Liu, J., Swaminathan, G., Xu, P., Li, Y., Baker, D.P., Peng, J., Lukacs, G.L.*, et al.* (2007). Site-specific ubiquitination exposes a linear motif to promote interferon-alpha receptor endocytosis. The Journal of cell biology *179*, 935-950.

Kurihara, L.J., Kikuchi, T., Wada, K., and Tilghman, S.M. (2001). Loss of Uch-L1 and Uch-L3 leads to neurodegeneration, posterior paralysis and dysphagia. Human molecular genetics *10*, 1963-1970.

Kurz, T., Pintard, L., Willis, J.H., Hamill, D.R., Gonczy, P., Peter, M., and Bowerman, B. (2002). Cytoskeletal regulation by the NEDD8 ubiquitin-like protein modification pathway. Science *295*, 1294-1298.

Kwon, J., Wang, Y.L., Setsuie, R., Sekiguchi, S., Sato, Y., Sakurai, M., Noda, M., Aoki, S., Yoshikawa, Y., and Wada, K. (2004). Two closely related ubiquitin C-terminal hydrolase isozymes function as reciprocal modulators of germ cell apoptosis in cryptorchid testis. The American journal of pathology *165*, 1367-1374.

Lee, C., and Goldberg, J. (2010). Structure of coatomer cage proteins and the relationship among COPI, COPII, and clathrin vesicle coats. Cell *142*, 123-132.

Lee, M.R., Lee, D., Shin, S.K., Kim, Y.H., and Choi, C.Y. (2008). Inhibition of APP intracellular domain (AICD) transcriptional activity via covalent conjugation with NEDD8. Biochemical and biophysical research communications *366*, 976-981.

Leidecker, O., Matic, I., Mahata, B., Pion, E., and Xirodimas, D.P. (2012). The ubiquitin E1 enzyme UBE1 mediates NEDD8 activation under diverse stress conditions. Cell cycle *11*, 1142-1150.

Leidecker, O., and Xirodimas, D.P. (2012). Isolation of NEDDylated proteins in human cells. Methods in molecular biology *832*, 133-140.

Leon, S., and Haguenauer-Tsapis, R. Immunoprecipitation of yeast proteins in denaturing conditions for analysis of ubiquitylation—Method 1 (Rubicon).

Liao, M., Zgoda, V.G., Murray, B.P., and Correia, M.A. (2005). Vacuolar degradation of rat liver CYP2B1 in Saccharomyces cerevisiae: further validation of the yeast model and structural implications for the degradation of mammalian endoplasmic reticulum P450 proteins. Molecular pharmacology *67*, 1460-1469.

Lim, K.L., and Tan, J.M. (2007). Role of the ubiquitin proteasome system in Parkinson's disease. BMC biochemistry *8 Suppl 1*, S13.

Liu, C., Apodaca, J., Davis, L.E., and Rao, H. (2007). Proteasome inhibition in wild-type yeast Saccharomyces cerevisiae cells. BioTechniques *42*, 158, 160, 162.

Madura, K., Dohmen, R.J., Varshavsky, A. (1993). N-recognin/Ubc2 interactions in the N-end rule pathway. JBC 268, 12046-12054.

Mendoza, H.M., Shen, L.N., Botting, C., Lewis, A., Chen, J., Ink, B., and Hay, R.T. (2003). NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins. The Journal of biological chemistry *278*, 25637-25643.

Milhollen, M.A., Traore, T., Adams-Duffy, J., Thomas, M.P., Berger, A.J., Dang, L., Dick, L.R., Garnsey, J.J., Koenig, E., Langston, S.P., *et al.* (2010). MLN4924, a NEDD8-activating enzyme inhibitor, is active in diffuse large B-cell lymphoma models: rationale for treatment of NF-{kappa}B-dependent lymphoma. Blood *116*, 1515-1523.

Mishra, P., Volety, S., Rao Ch, M., and Prabha, C.R. (2009). Glutamate64 to glycine substitution in G1 beta-bulge of ubiquitin impairs function and stabilizes structure of the protein. Journal of biochemistry *146*, 563-569.

Miyoshi, Y., Nakayama, S., Torikoshi, Y., Tanaka, S., Ishihara, H., Taguchi, T., Tamaki, Y., and Noguchi, S. (2006). High expression of ubiquitin carboxy-terminal hydrolase-L1 and -L3 mRNA predicts early recurrence in patients with invasive breast cancer. Cancer science *97*, 523-529.

Nagano, T., Hashimoto, T., Nakashima, A., Kikkawa, U., and Kamada, S. (2012). X-linked inhibitor of apoptosis protein mediates neddylation by itself but does not function as a NEDD8-E3 ligase for caspase-7. FEBS letters *586*, 1612-1616.

Nam, M.J., Madoz-Gurpide, J., Wang, H., Lescure, P., Schmalbach, C.E., Zhao, R., Misek, D.E., Kuick, R., Brenner, D.E., and Hanash, S.M. (2003). Molecular profiling of the immune response in colon cancer using protein microarrays: occurrence of autoantibodies to ubiquitin C-terminal hydrolase L3. Proteomics *3*, 2108-2115.

Nillegoda, N.B., Theodoraki, M.A., Mandal, A.K., Mayo, K.J., Ren, H.Y., Sultana, R., Wu, K., Johnson, J., Cyr, D.M., Caplan, A.J. (2010) Ubr1 and Ubr2 Function in a Quality Control Pathway for Degradation of Unfolded Cytosolic Proteins. MBOC 12(13), 2102-2116.

Nowrocki, S.T., Griffin, P., Kelly K.R., Carew, J.S. (2012). MLN4924: a novel first-in-class inhibitor of NEDD8-activating enzyme for cancer therapy. Expert Opin Investig Drugs 10, 1563-1573.

Oved, S., Mosesson, Y., Zwang, Y., Santonico, E., Shtiegman, K., Marmor, M.D., Kochupurakkal, B.S., Katz, M., Lavi, S., Cesareni, G., *et al.* (2006). Conjugation to NEDD8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases. The Journal of biological chemistry *281*, 21640-21651.

Pickart, C.M., and Eddins, M.J. (2004). Ubiquitin: structures, functions, mechanisms. Biochimica et biophysica acta *1695*, 55-72.

Popoff, V., Adolf, F., Brugger, B., and Wieland, F. (2011). COPI budding within the Golgi stack. Cold Spring Harbor perspectives in biology *3*, a005231.

Rabut, G., and Peter, M. (2008). Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series. EMBO reports *9*, 969-976.

Reverter, D., Wu, K., Erdene, T.G., Pan, Z.Q., Wilkinson, K.D., and Lima, C.D. (2005). Structure of a complex between NEDD8 and the Ulp/Senp protease family member Den1. Journal of molecular biology *345*, 141-151.

Reyes-Turcu, F.E., Ventii, K.H., and Wilkinson, K.D. (2009). Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annual review of biochemistry *78*, 363-397.

Russell, R.C., and Ohh, M. (2008). NEDD8 acts as a 'molecular switch' defining the functional selectivity of VHL. EMBO reports *9*, 486-491.

Ryu, J.H., Li, S.H., Park, H.S., Park, J.W., Lee, B., and Chun, Y.S. (2011). Hypoxia-inducible factor alpha subunit stabilization by NEDD8 conjugation is reactive oxygen species-dependent. The Journal of biological chemistry *286*, 6963-6970.

Sadis, S., Atienza, C., Jr., and Finley, D. (1995). Synthetic signals for ubiquitin-dependent proteolysis. Molecular and cellular biology *15*, 4086-4094.

Salton, M., Lerenthal, Y., Wang, S.Y., Chen, D.J., and Shiloh, Y. (2010). Involvement of Matrin 3 and SFPQ/NONO in the DNA damage response. Cell cycle *9*, 1568-1576.

Sano, Y., Furuta, A., Setsuie, R., Kikuchi, H., Wang, Y.L., Sakurai, M., Kwon, J., Noda, M., and Wada, K. (2006). Photoreceptor cell apoptosis in the retinal degeneration of UCHL3-deficient mice. The American journal of pathology *169*, 132-141.

Sarikas, A., Hartmann, T., and Pan, Z.Q. (2011). The cullin protein family. Genome biology *12*, 220.

Schmidt, M.W., McQuary, P.R., Wee, S., Hofmann, K., and Wolf, D.A. (2009). F-box-directed CRL complex assembly and regulation by the CSN and CAND1. Molecular cell *35*, 586-597.

Sela, N., Atir-Lande, A., and Kornitzer, D. (2012). Neddylation and CAND1 independently stimulate SCF ubiquitin ligase activity in Candida albicans. Eukaryotic cell *11*, 42-52.

Semenova, E., Wang, X., Jablonski, M.M., Levorse, J., and Tilghman, S.M. (2003). An engineered 800 kilobase deletion of UCHL3 and Lmo7 on mouse chromosome 14 causes defects in viability, postnatal growth and degeneration of muscle and retina. Human molecular genetics *12*, 1301-1312.

Setsuie, R., Sakurai, M., Sakaguchi, Y., and Wada, K. (2009a). Ubiquitin dimers control the hydrolase activity of UCH-L3. Neurochemistry international *54*, 314-321.

Setsuie, R., Suzuki, M., Kabuta, T., Fujita, H., Miura, S., Ichihara, N., Yamada, D., Wang, Y.L., Ezaki, O., Suzuki, Y.*, et al.* (2009b). Ubiquitin C-terminal hydrolase-L3-knockout mice are resistant to diet-induced obesity and show increased activation of AMP-activated protein kinase in skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *23*, 4148-4157.

Setsuie, R., Suzuki, M., Tsuchiya, Y., and Wada, K. (2010). Skeletal muscles of UCHL3 knockout mice show polyubiquitinated protein accumulation and stress responses. Neurochemistry international *56*, 911-918.

Seufert, W., and Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. The EMBO journal *9*, 543-550.

Shabek, N., and Ciechanover, A. (2010). Degradation of ubiquitin: the fate of the cellular reaper. Cell cycle *9*, 523-530.

Siergiejuk, E., Scott, D.C., Schulman, B.A., Hofmann, K., Kurz, T., and Peter, M. (2009). Cullin neddylation and substrate-adaptors counteract SCF inhibition by the CAND1-like protein Lag2 in Saccharomyces cerevisiae. The EMBO journal *28*, 3845-3856.

Skaar, J.R., Arai, T., and DeCaprio, J.A. (2005). Dimerization of CUL7 and PARC is not required for all CUL7 functions and mouse development. Molecular and cellular biology *25*, 5579-5589.

Skaar, J.R., Florens, L., Tsutsumi, T., Arai, T., Tron, A., Swanson, S.K., Washburn, M.P., and DeCaprio, J.A. (2007). PARC and CUL7 form atypical cullin RING ligase complexes. Cancer research *67*, 2006-2014.

Smith, M.A., Maris, J.M., Gorlick, R., Kolb, E.A., Lock, R., Carol, H., Keir, S.T., Reynolds, C.P., Kang, M.H., Morton, C.L.*, et al.* (2012). Initial testing of the investigational

NEDD8-activating enzyme inhibitor MLN4924 by the pediatric preclinical testing program. Pediatric blood & cancer *59*, 246-253.

Smith, P.K., R.I.Krohn, G.T.Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D. C. Klenk. (1985). Measurement of Protein Using Bicinchoninic Acid. Anal Biochem, 76-85.

Souphron, J., Waddell, M.B., Paydar, A., Tokgoz-Gromley, Z., Roussel, M.F., and Schulman, B.A. (2008). Structural dissection of a gating mechanism preventing misactivation of ubiquitin by NEDD8's E1. Biochemistry *47*, 8961-8969.

Staub, O., and Rotin, D. (2006). Role of ubiquitylation in cellular membrane transport. Physiological reviews *86*, 669-707.

Stickle, N.H., Chung, J., Klco, J.M., Hill, R.P., Kaelin, W.G., Jr., and Ohh, M. (2004). pVHL modification by NEDD8 is required for fibronectin matrix assembly and suppression of tumor development. Molecular and cellular biology *24*, 3251-3261.

Stolz, A. (2011). Endoplasmic Reticulum Associated Protein Degradation (ERAD): The Function of Dfm1 and Other Novel Components of the Pathway (Institut fur Biochemie der Universitat Stuttgart).

Upadhya, S.C., and Hegde, A.N. (2007). Role of the ubiquitin proteasome system in Alzheimer's disease. BMC biochemistry *8 Suppl 1*, S12.

Wada, H., Kito, K., Caskey, L.S., Yeh, E.T., and Kamitani, T. (1998). Cleavage of the C-terminus of NEDD8 by UCH-L3. Biochemical and biophysical research communications *251*, 688-692.

Wada, H., Yeh, E.T., Kamitani, T. (2000). A dominant-negative UBC12 mutant sequesters NEDD8 and inhibits NEDD8 conjugation in vivo. JBC 275(22), 17008-17015.

Walden, H., Podgorski, M.S., Huang, D.T., Miller, D.W., Howard, R.J., Minor, D.L., Jr., Holton, J.M., and Schulman, B.A. (2003). The structure of the APPBP1-UBA3-NEDD8-ATP complex reveals the basis for selective ubiquitin-like protein activation by an E1. Molecular cell *12*, 1427-1437.

Watson, I.R., Blanch, A., Lin, D.C., Ohh, M., and Irwin, M.S. (2006). Mdm2-mediated NEDD8 modification of TAp73 regulates its transactivation function. The Journal of biological chemistry *281*, 34096-34103.

Watson, I.R., Irwin, M.S., and Ohh, M. (2011). NEDD8 pathways in cancer, Sine Quibus Non. Cancer cell *19*, 168-176.

Watson, I.R., Li, B.K., Roche, O., Blanch, A., Ohh, M., and Irwin, M.S. (2010). Chemotherapy induces NEDP1-mediated destabilization of MDM2. Oncogene *29*, 297-304.

Wei, N., and Deng, X.W. (2003). The COP9 signalosome. Annual review of cell and developmental biology *19*, 261-286.

Whitby, F.G., Xia, G., Pickart, C.M., and Hill, C.P. (1998). Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes. The Journal of biological chemistry *273*, 34983-34991.

Wood, M.A., Kaplan, M.P., Brensinger, C.M., Guo, W., and Abel, T. (2005). Ubiquitin C-terminal hydrolase L3 (UCHL3) is involved in working memory. Hippocampus *15*, 610-621.

Wu, K., Yamoah, K., Dolios, G., Gan-Erdene, T., Tan, P., Chen, A., Lee, C.G., Wei, N., Wilkinson, K.D., Wang, R*., et al.* (2003). DEN1 is a dual function protease capable of processing the C terminus of NEDD8 and deconjugating hyper-neddylated CUL1. The Journal of biological chemistry *278*, 28882-28891.

Xia, Z., Webster, A., Du, F., Piatkov, K., Ghislain, M., and Varshavsky, A. (2008). Substrate-binding sites of UBR1, the ubiquitin ligase of the N-end rule pathway. J Biol Chem *283*, 24011-24028.

Xirodimas, D.P., Saville, M.K., Bourdon, J.C., Hay, R.T., and Lane, D.P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell *118*, 83-97.

Xirodimas, D.P., Sundqvist, A., Nakamura, A., Shen, L., Botting, C., and Hay, R.T. (2008). Ribosomal proteins are targets for the NEDD8 pathway. EMBO reports *9*, 280-286.

Ye, Y., Akutsu, M., Reyes-Turcu, F., Enchev, R.I., Wilkinson, K.D., and Komander, D. (2011). Polyubiquitin binding and cross-reactivity in the USP domain deubiquitinase USP21. EMBO reports *12*, 350-357.

Zerella, R., Evans, P.A., Ionides, J.M., Packman, L.C., Trotter, B.W., Mackay, J.P., and Williams, D.H. (1999). Autonomous folding of a peptide corresponding to the N-terminal beta-hairpin from ubiquitin. Protein science : a publication of the Protein Society *8*, 1320-1331.

Zhang, Z., and Carmichael, G.G. (2001). The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. Cell *106*, 465-475.

Zheng J, Yang X, Harrell JM, Ryzhikov S, Shim EH, et al. (2002) CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. Mol Cell 10: 1519–1526.